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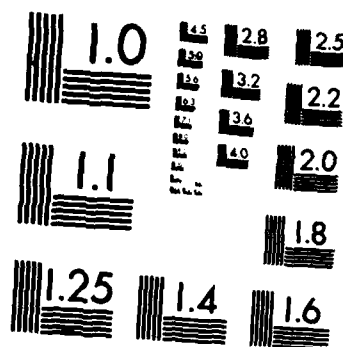
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Institute for Applied Microbiology
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October 23, 1986

Dr. Eli D. Schmell
Program Manager, Molecular Biology
Code 1141MB
Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5000

Dear Eli,

Here is our annual report on the corrosion grant together with some preprints and some illustrations you could make into overheads or slides if you want to talk about corrosion. Bearnie told me that you might be proposing a microbially facilitated corrosion initiative around Brenda Little. Please let me help you as that would be wonderful.

We are very excited. It seems that next to us in our new laboratory on the Pellissippi technology corridor is the Computer Technology and Imaging Co. It turns out that they make the positron imaging apparatus for Siemans that enables detection of labeled isotopes (made in their handy little cyclotron) with very short half lives that are used in humans where they have a resolution of 2 mm. They are talking to us about microns—we may be able to watch living microcolonies do their chemistry non-destructively! Behind us in the enantiomorphic building is Pellissippi International, Inc., the newest company of G. Sam Hurst who has a laser technique that increases IR sensistivity by 5-orders of magnitude and has immediate access to the ORNL scanning tunneling electron microscope and wants to do a biological project. I fell into a gold mine! Our new lab is beautiful although it is currently devoid of lab benches, sinks and hoods. Supposidly that will come with time. You need to site visit us!

Good to see you and the gang at Belmont but I was sorry to miss Warren. Give him my best. We may be able to do fantastic things with Mike Silverman's adhesion mutants.

Sincerely,

David C. White M.D., Ph.D.
UTK/ORNL Distinguished Scientist

C C. Ph.D. > Ha less

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Research is directed to develop methods by which the mechanisms of microbially facilitated corrosion (MFC) operate. Mechanisms such as the formation or utilization of hydrogen, the generation of mineral or organic acids, the generation of effective metal chelators, the active reduction of metals with the creation of areas of differential cathodic activity are postulated. Sensitive methods for microbial community structure, metabolic activities, and nutritional status have been developed utilizing signature lipid biomarker that allow quantitative defination of microbial consortia. These have been correlated with nondestructive Fourier transform spectroscopy (FT/IR) and electrochemical measures of corrosion potential. With these the potential for microbial consortia to form microcolonies have been shown to allow enhanced MFC in which multiple physiologic types of microbes can attack metals by several mechanisms. Aerobic polymer secreting heterotrophs form microcolonies and create anaerobic microniches in aerobic bulk water that allow acid secreting or hydrogen utilizing anaerobes to function and induce MFC.

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# PROGRESS REPORT, N000014-83-K-0056, MOLECULAR MECHANISMS OF MICROBIALLY FACILITATED CORROSION

David C. White, Institute for Applied Microbiology, 10515 Research Drive, Suite 300, Bldg. #1, Knoxville, TN 37932-2567 (phone 615-675-9520)

SUMMARY OF PROJECT GOALS:
1) To correlate corrosion to the specific activities of microbes, consortia of microbes and their extracellular products to discover mechanisms of microbially facilitated corrosion (MFC); 2) In terms of these mechanisms of MFC to define conditions that increase or decrease corrosion; 3) To increase the specificity and sensitivity of methods to define the community structure, nutritional status and metabolic activities of MFC consortia by gas chromatography/mass spectrometry (GC/MS) to correlate with Fourier transform infrared spectroscopy (FT/IR); 4) To measure effects of bulk media composition, surface chemistry, and topology on the MFC potential of various monocultures and biofilms; 5) To develop methods to measure corrosion on the scale of microcolonies (microns); and 6) To quantitatively test countermeasures on attachment and proliferation of corroding biofilms.

RECENT ACCOMPLISHMENTS:
Published work from this grant include a review showing the applicability of the phospholipid ester-linked fatty acid (PLFA) analysis to defining the microbial community structure. Other lipid measures of microbial community nutritional status based on the rates of formation of the endogenous storage lipid poly beta-hydroxybutyrate (PHB) and the metaboilic activity from rates of incorportion into lipids and DNA are detailed in the analysis of toxicity assessment (1). The structural detail of the PLFA analysis was greatly increased with the development of the dimethyldisulfide adducts for GC and CG/MS (8). With this derivitization we were able to demonstrate signature PLFA in two types of methane oxidizing bacteria (2) and then to show that type II methanotrophs increase dramatically in sediments exposed to natural gas. This consortia that develops with exposure to natural gas and air rapidly degrades halogenated hydrocarbons (4). This work which was a by- product of the corrosion research is the basis of in situ rectification of contaminated ground water. We examined the mineral acid secreting bacteria as MFC agents and were able to show an incredible array of signature PLFA in the acid producing Thiobacilli (5). We were then able to utilize these biomarkers to establish the role of these acid producing Thiobacilli in the biodegredation of concrete (6). This work forms the basis of a biotest system for the development of MFC resistant concrete (7). This system is utilized extensively in West Germany and the PLFA analysis has greatly increased the sensitivity. We continued our work with the sulfate-reducing bacteria (SRB) and were able to define signature lipid biomarkers for the fatty acid utilizing Desulfobacter type SRB's (3). With this marker in hand We were able to establish that in most environments it is the Desulfobacter not the Desulfovibrio that are the important SRBs. It was then possible to provide these organisms and this insight to D. Pope who utilized fluoro-antibodies to test the correlaton of corrosion with the biomass of SRB's in many field samples and it is becomming increasingly apparent that the correlation is very poor. The presence of total microbial consortia apparently gives much better correlations with MFC. Consequently we demonstrated that consortia were more effective in MFC than monocultures. To initiate studies of MFC consortia we incubated stainless steel coupons in a B. Little type galvanic corrosion cell and showed that it was possible to set

up a consortium of a facultative <u>Vibrio</u> and the anaerobe <u>Desulfobacter</u> that
was much more corrosive than either monoculture. Furthermore it was possible
to create the consortia in aerated sea water even though the <u>Desulfobacter</u> is
a strict anaerobe (see presentations A). We were able to establish that a
consortium can create anaerobic microniches in aerated systems that
facilitate MFC. With the signature biomarker techniques we have developed we
now hope to explore consortium structure and activity in much greater detail.
Work supported by this proposal enabled us to develop and test a method by
which the particulate composition of the bulk water can be examined. We
showed a hexane:isopropanol extraction of PLFA was quantitative and did not
disturb Nuclepore membranes (Guckert, Ph.D. thesis). This makes possible
studies of the effects of treatments on the recruitment of specific microbes
from the particulates or the pelagic bacterial suspension that can facilitate
MFC (for example where do the anaerobes come from in aerobic seawater). Our
work has shown the utility of the FT/IR in the nondestructive analysis of
biofilms (Presentations B). This combined with the PLFA methodology reviewed
in (1) means that the methods now exist to examine the microbial consortia at
the micron scale of the colonies. What is now necessary is the development
of methods for quantitatively demonstrating the spacial concordance of
corrosion and microbial consortial activities on the scale of microbes.

PLANS FOR NEXT YEAR:
        Dowling is spending the fall with Dr. J. Guezennec at IFREMER in
Brest, France where he will be utilizing one of our B. Little type galvanic
cell chemostats in their extensive marine test systems. He will be teaching
them the signature biomarker GC/MS techniques and they will train him in the
use and and intrepretation of alternating current impedance cell monitoring
for biofilms and corrosion. D. Nivens is taking electrochemistry as part of
his Ph.D. program in analytical chemistry so we are bolstering our
understanding of how to measure corrosion in the presence of irregular
biofilms. The studies of B. Little make it clear that the use of the
standard polarization potentiometry which assumes a uniform surface is not
accurate in problems fo MFC. A new Ph.D. student, M. Franklin, is learning
the lipid techniques so he can combine his microbiological skills with
fastidious anaerobes with chemical probes.
        Initially this year we plan to concentrate on Iron reducing and
oxidizing bacteria which we have isolated and characterized signature PLFA
from corroding well casings. There seems to be developing a whole system of
microbes based on iron that is somewhat less active than nitrate but of a
higher potential than sulfate. We feel we can use their unusual morphology
to study new mechanisms for MFC. We also plan to utilize the HPLC methods we
have developed for Archaebacterial lipids to study the role of these
organisms in MFC. We will also be developing measures for the microbes that
produce organic acids as metabolic end products. We plan to try and directly
measure the loss of metal from sputter coated coupons using the excellent SCM
and EDAX facilities at UTK. We are planning to be able to greatly increase
the sensitivity of the GC/MS analysis by using various funds to purchase an
EXTREL GC/CINIMS that will increase the sensitivity of the PLFA analysis by 5
orders of magnitude using the methods we developed in Sweden in 1985. We are
also planning to use part of the equipment budget from this grant to purchase
a good microscopic attachment for the FT/IR so we can examine microcolonies
nondestructively prior to the SCM-EDAX analysis. We are actively negotiating
to utilize the assay systems we have developed with an industrial corporation
to test chemical countermeasures on shifting the microbial community stucture
as it relates to MFC.

2

## DATA SYNOPSIS

(a) Publications in reviewed literature:

1. White, D. C. 1986. Environmental effects testing with quantitative microbial analysis: Chemical signatures correlated with in situ biofilm analysis by FT/IR. Toxicity Assessment 1: 315-338.
2. Nichols, P. D., G. A. Smith, C. P. Antworth, R. S. Hanson, and D. C. White. 1985. Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for the methane-oxidizing bacteria. F. E. M. S. Microbiol. Ecology 31: 327-335.
3. Dowling, N. J. E., F. Widdel, and D. C. White. 1986. Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide forming bacteria. J. Gen. Microbiol. 132: 1815-1825.
4. Nichols, P. D., J. M. Henson, C. P. Antworth, J. Parsons, J. T. Wilson and D. C. White. 1986. Detection of a microbial consortium including type II methanotrophs by use of phospholipid fatty acids in an aerobic halogenated hydrocarbon-degrading soil column enriched with natural gas. Environ. Toxicol. Chem. 5: in press.
5. Kerger, B. D., P. D. Nichols, C. A. Antworth, W. Sand, E. Bock, J. C. Cox, T. A. Langworthy, and D. C. White. 1986. Signature fatty acids in the polar lipids of acid producing Thiobacilli: methoxy, cyclopropyl, alpha-hydroxy-cyclopropyl and branched and normal monoenoic fatty acids. F. E. M. S. Microbiol. Ecology 38: 67-77.
6. Kerger, B. D., P. D. Nichols, W. Sand, E. Bock, and D. C. White. 1986. Association of acid producing Thiobacilli with degradation concrete: analysis by "signature" fatty acids from the polar lipids and lipopolysaccharide. J. Industrial Microbiol. 1: in press.
7. Sand, W., E. Bock, and D. C. White. 1986. Biotest system for rapid evaluation of concrete resistance to sulfur oxidizing bacteria. Materials Performance 24: in press.
8. Nichols, P. D., J. B. Guckert, and D. C. White. 1986. Determination of microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. J. Microbiol. Methods 5: 49-55.

(b) Awarded UTK/ORNL Distinguished Scientist Position July, 1986

(c) Presentations:

A.  Dowling, N. J. E., J. Guezennec, and D. C. White. 1986. Facilitation of corrosion of stainless steel exposed to aerobic seawater by microbial biofilms containing both facultative and absolute anaerobes. In Microbial Problems in the Offshore Oil Industry, Proceedings of Conference, Aberdeen, Scotland.

B.  White, D. C. 1986. Non-destructive biofilm analysis by Fourier transform spectroscopy (FT/IR). Proceed. Fifth Int. Congress of Microbial Ecology. Ljubljana, Yugoslavia, August 1986.

(d) Ph. D. Graduate Students supported:

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David E. Nivens, BA. Chemistry UTK (white male, USA)
Michael J. Franklin, Microbiology UTK (white male, USA)

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MICRO 3/3

# BIOTEST SYSTEM FOR RAPID EVALUATION OF CONCRETE RESISTANCE TO SULFUR

## OXIDIZING BACTERIA

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# ABSTRACT

Concrete exposed to sewage or industrial waste in the presence of air and inorganic reduced sulfur compounds often degrades rapidly. Sulfur oxidizing bacteria of the genus Thiobacillus which generate sulfuric acid as end product of their metabolism play an important role in this process. To evaluate the resistance of concrete to the activity of these microorganisms, a specially designed hydrogen sulfide chamber containing concrete test blocks was built. In this chamber, temperature, humidity, hydrogen sulfide concentration and exposure to aerosols of different thiobacilli are controlled. Experiments in this chamber show the rate of concrete degradation is accelerated so that corrosion that required at least 5 years in sewer systems was reproducibly demonstrated in 9 months. With this system the rates of degradation corresponding to a weight loss of between 1 and 10% were shown to correlate most closely to densities of between $10^6$ to $10^8$ cells/$cm^2$ of the bacterium T. thiooxidans on the surface of the concrete test specimens. Specific polar lipid components in the membranes of the thiobacilli can be utilized to monitor the number of these organisms on the surfaces of corroding concrete.

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## INTRODUCTION

The utilization of the versatile and relatively inexpensive building material concrete in certain environments can lead to severe corrosion problems. In the presence of a reduced form of sulfur, oxygen, nitrogen, and a carbon source that can be carbon dioxide, reduced sulfur oxidizing microorganisms can generate sulfuric acid. In the presence of the sulfuric acid gypsum can be created from the calcium hydroxides and carbonates with often disastrous weakening of the structure. This problem has been noted primarily in concrete sewer lines, particularly in warm and moderate climates [1-4]. Parker in 1945 [5] described a biologically mediated corrosion mechanism for the Melbourne, Australia, sewer system. Microbially generated hydrogen sulfide is transported to the walls of the sewer pipes and converted to sulfur. This sulfur is oxidized to sulfuric acid by the metabolism of the thiobacilli. This theory has been confirmed by our work [4,6-12]. In the course of study of sulfuric acid attack on the sewer network of Hamburg (FRG) an in-situ study of the thiobacilli that were involved in the biocorrosion process proved them to be Thiobacillus intermedius, T. novellus, T. neopolitanus, and T. thiooxidans [6].

To study the corrosion process under controlled conditions a chamber was constructed in which the temperature, humidity, hydrogen sulfide concentration, and exposure to aerosols of specific mixtures of these thiobacilli was accurately controlled. In this chamber the corrosion was reproducibly accelerated so that a process taking more than 5 years in the field could be observed in 9 months. The test system thus became an excellent vehicle to monitor the resistance of different types of concrete as well as to monitor the effects of cell numbers and species of thiobacilli on rates of corrosion [13]. Preliminary evidence is also presented showing the

long process of viable counting of cultured thiobacilli isolated from the
concrete surfaces can be supplemented or replaced by quantitative
measurements of the "signature" components of polar lipids of these
organisms.

EXPERIMENTAL PROCEDURE

Controlled exposure chamber.

A stainless steel chamber was constructed in which 32 concrete specimens
60 cm x 11 cm x 7 cm (h x w x d) in size that were scored into 1.8 cm squares
were placed on end with the base standing in 10 cm of water. The temperature
of the water was maintained at 30°C with the pH held at 7.0 by means of an
automatic titration unit. The air space above the water was maintained at 30°C,
at 100% humidity, with 10 ± 1 ppm hydrogen sulfide as monitored by a
gas chromatograph [4]. The blocks were periodically sprayed with cultures of
thiobacilli that had been isolated from the sewer system. The pH at the
concrete surface was monitored by firmly attached pH strips on the concrete
specimens. The strips were replaced every 2 weeks. The test apparatus is
diagrammed in Figure 1. The concrete test specimens and chamber are
illustrated in Figure 2.

Monitoring the corrosion

After a 3 month inoculation period each specimen was sampled every 90
days for 3 to 4 periods. The surface cubes were broken off the concrete test
samples, transferred to sterile bottles containing 50 ml of sterile washing
solution (see below), and incubated in flasks rotated at 200 RPM on a shaker
for 30 min. The resulting suspensions were used for dilution series, from
which selective media (see below) for thiobacilli, bacterial heterotrophs,
and fungi were inoculated. The cultural evaluation took 4 weeks. The
weight loss of the cubes was monitored at the start and end of the
experiments. After shaking the cubes for 2 hours in sterile washing solution

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both the cubes and the dried washing solution were weighed. The loss of weight equalled the weight of the dried corrosion products found in the washing solution divided into the sum of the dried corrosion products and dried cubes.

### Monitoring bacterial growth

The most probable number (MPN) technique was utilized to establish the microbes involved with the corrosion. For T.intermedius and T. novellus the medium contained $Na_2S_2O_3-5H_2O$, 5.0 g/l; $CaCl-2H_2O$, 0.13 g/l; $NH_4Cl$, 1.0 g/l; $MgSO_4-7H_2O$, 1.02 g/l; $KH_2PO_4$, 0.4 g/l; $K_2HPO_4$, 0.6 g/l; trace metal solution: (5 ml/l of EDTA, 50 g/l; $ZnSO_4-7H_2O$, 2.2 g/l; $CaCl_2$, 5.5 g/l; $MnCl_2-4H_2O$, 5.06 g/l; $FeSO_4-6H_2O$, 5.0 g/l; $(NH_4)_6Mo_7O_{24}$, 1.1 g/l; $CuSO_4-5H_2O$, 1.57 g/l; $CoCl_2-6H_2O$, 1.61 g/l; in 1.0 l distilled water), ferric-EDTA, 2 mg/l; biotin, 24.4 mg/l; in 1.0 l distilled water with a final pH of 6.5. For T. neopolitanus the medium contained $Na_2S_2O_3-5H_2O$, 10 g/l; $MgSO_4-7H_2O$, 0.8 g/l; $NH_4Cl$, 0.4 g/l; trace metal solution, 10 ml/l; $KH_2PO_4$, 4.0 g/l; $K_2HPO_4$, 4.0 g/l; in 1.0 l distilled water. For T. thiooxidans the medium contained $Na_2S_2O_3-5H_2O$, 10.0 g/l; $KH_2PO_4$, 2.0 g/l; CaCl, 1.0 g/l; $MgSO_4-7H_2O$, 0.2 g/l; $(NH_4)_2SO_4$, 0.1 g/l; $CaCl_2-2H_2O$, 0.04 g/l; $FeCl_3-6H_2O$, 0.02 g/l; and $MnSO_4-H_2O$, 0.015 g/l in 1.0 l distilled water. The washing solution was the T. neapolitanus medium without thiosulfate. The media were sterilized for 30 min at $110^\circ C$.

The cell counts were done on serial dilutions in steps of 1 to 10. Five culture tubes with 2.5 ml of each medium were inoculated with 0.5 ml of the serial dilution steps and incubated on a rotary incubator at 30 $^\circ C$ for aeration. The tests were evaluated after 3 weeks of incubation. Tests for T. neapolitanus and T. thiooxidans were considered positive if the pH of the medium was below 4.0 and 2.0 respectively. To differentiate between T. intermedius/novellus and T. neapolitanus which both grow on the T.

<u>intermedius</u> medium, each test tube with turbidity and a pH below pH 4.0 was streaked on <u>T. intermedius/novellus</u> agar (salts plus 1.5% agar) and incubated at 30°C for 1 week. The test was considered positive for <u>T. intermedius/novellus</u> if the colonies were transparent, and yellowish, and negative if the colonies were opaque and white (<u>T. neapolitanus</u>).

Heterotrophic aerobic bacteria were scored after growth on agar plates containing DEV-gelatin agar (Merck, FRG). Fungi were counted after growth on Sabouraud-Maltose agar (Merck, FRG).

### Analysis for signature lipids

Specimens of corroded concrete and monocultures of the thiobacilli were extracted with a one phase chloroform-methanol solvent and the lipids fractionated on silicic acid columns [14,15]. The polar lipid fraction recovered in methanol was subjected to mild alkaline methanolysis and the products partitioned against water. The water portion was analyzed for phosphate (total extractable phospholipid) [14]. The lipid portion was fractionated into the acyl fatty acid methyl esters and hydroxy fatty acid methyl esters by thin layer chromatography, the esters recovered and analyzed by capillary gas liquid chromatography and with structural confirmation by mass spectrometry [16].

## RESULTS AND DISCUSSION

### Corrosion and surface pH of concrete

Three types of concrete show different responses to the corrosive activities of the thiobacilli (Figure 3). All specimens had an initial pH of between 9 to 11. With a resistant Portland cement shown In the upper panel of Figure 3 the pH decreased within 120 days to between 2.0 ± 0.3 and remained constant until the end of the experiment. The middle panel of Figure 3 shows a Portland cement of intermediate resistance. The initial pH remained

constant for 70 days, decreased to pH values betweeen 2 and 3 in the next 45 days and then remained at that pH until the end of the experiment. The lower panel shows the response of blast furnace type cement with the poorest resistance to the microbial corrosion. The pH decreased to pH 3 within 50 days and continued to fall to a pH of 1.0.

### Cell counts of thiobacilli and corrosion of concrete

The cell counts of the three major groups of thiobacilli show highest levels in the most rapidly degrading concrete (lower panel, Figure 3). Although the lowest levels of T. intermedius/novellus appear to be associated with the most resistant concrete (upper panel, Figure 3), the best correlations between rates of degradation of the concrete and thiobacilli are with the most powerful acid generating species T. thiooxidans. This is illustrated in the data of Table 1.

The plate counts of heterotrophic aerobic bacteria and fungi were independent of the concrete specimen tested.

### Potential of signature lipid analysis of thiobacilli

Assay of the thiobacilli from the dilution tubes and culture plates requires at least 4 weeks and cannot distinguish between T. intermedius and T. novellus. Recently the use of chemical assays of the lipid components of microbial consortia has been shown to provide a quantitative measure of the biomass and community structure without the necessity of isolation of the organisms from the growth substrate or culture of the organisms once they are isolated [17]. The early work of Shively [18,19] suggested that the lipids of the thiobacilli were sufficiently unusual to serve as signatures of these organisms. Work for our laboratory has shown that the thiobacilli polar lipids contain ester-linked and amide-linked fatty acids with unusual structures such as monoenoic 15, 16 and 17 carbon fatty acids with unsaturations at 5, 6, 7, 8, and 9 carbons from the methyl end of the

molecules [20]. Most organisms have the unsaturation at a single position usually at the 7 or 9 position from the methyl end. These organisms also contain unusually large proportions of cyclopropane fatty acids with the three membered ring between 7 and 8 in the 17 carbon and between 8 and 9 in the 19 carbon atom fatty acids. These lipids also contain unusual 2 hydroxyl monounsaturated, 10 and 11 methyl branched, 2 hydroxyl cyclopropane, 10 through 13 methoxy saturated, 10 and 13 hydroxy saturated fatty acids. These fatty acids occur in proportions that enable the individual species to be differentiated from each other [20]. The unusual polar lipid fatty acids and the bound fatty acids from the lipopolysaccharide enable the acid producing thiobacilli to be identified in samples from the test specimens and corroded concrete sewer samples from the field [21].

CONCLUSIONS

It has proved possible to correlate in sewer pipes, in the field,[11] and on concrete specimens in a strictly controlled hydrogen sulfide test chamber [12] that the degree of concrete degradation directly correlates with the numbers of Thiobacillus thiooxidans. These organisms depress the pH of the surface of the concrete to values between 1 and 3 by their excretion of sulfuric acid. The development of a biochemical assay for the thiobacilli allows greater insight into the relationship between metabolic activity of the organisms and the degradation of concrete. The development of this test chamber which provides reproducible exposures to the biodegradative activity of the Thiobacilli in reasonable timespans has led to a biotest facility for this most versatile building material.

ACKNOWLEDGEMENTS

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Table 1 Relationship between biodegradation of concrete and number of Thiobacillus thiooxidans.

| Corrosion Grade | Loss of Weight % of Total | Number of T. thiooxidans Logarithm cells/cm$^2$ | Number Analyzed |
|---|---|---|---|
| Negligible | 0.7 ± 0.5 | 6.8 ± 0.6 | 3 |
| Medium | 2.3 ± 1.7 | 7.1 ± 0.7 | 6 |
| Great | 5.8 ± 2.9 | 7.7 ± 0.5 | 5 |

FIGURE LEGENDS

Figure 1. Diagram of the constant temperature, humidity, and hydrogen sulfide "Biotest" chamber.

Figure 2. Photograph of the "Biotest" chamber showing the concrete samples in place with the surface pH test strips.

Figure 3. Profile of the pH (X) and logarithms of the cell numbers of <u>T. intermedius/novellus</u> (1), <u>T.</u> <u>neapolitanus</u> (2), and <u>T.</u> <u>thiooxidans</u> (3) measured after 270 days in the test chamber. Upper panel: resistant Portland cement; middle panel: intermediate resistant Portland cement; and lower panel: blast-furnace cement of poorest resistance.
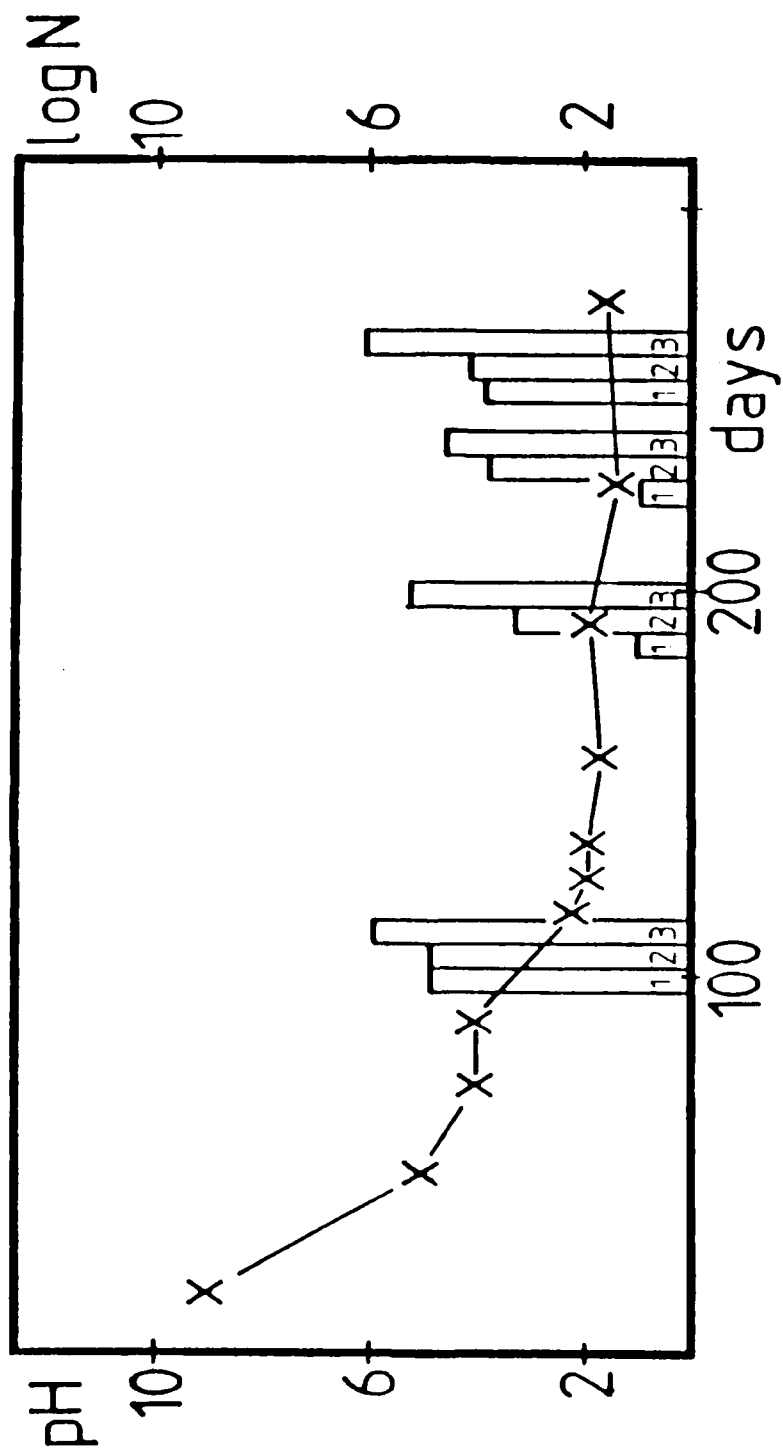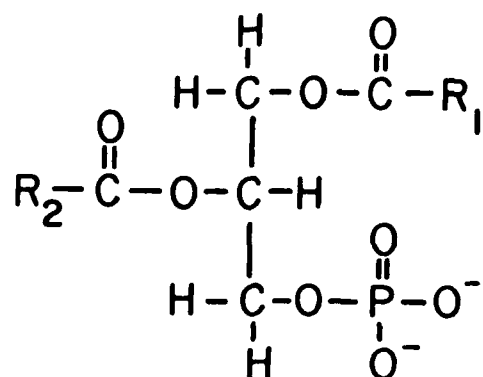
Fig. 1
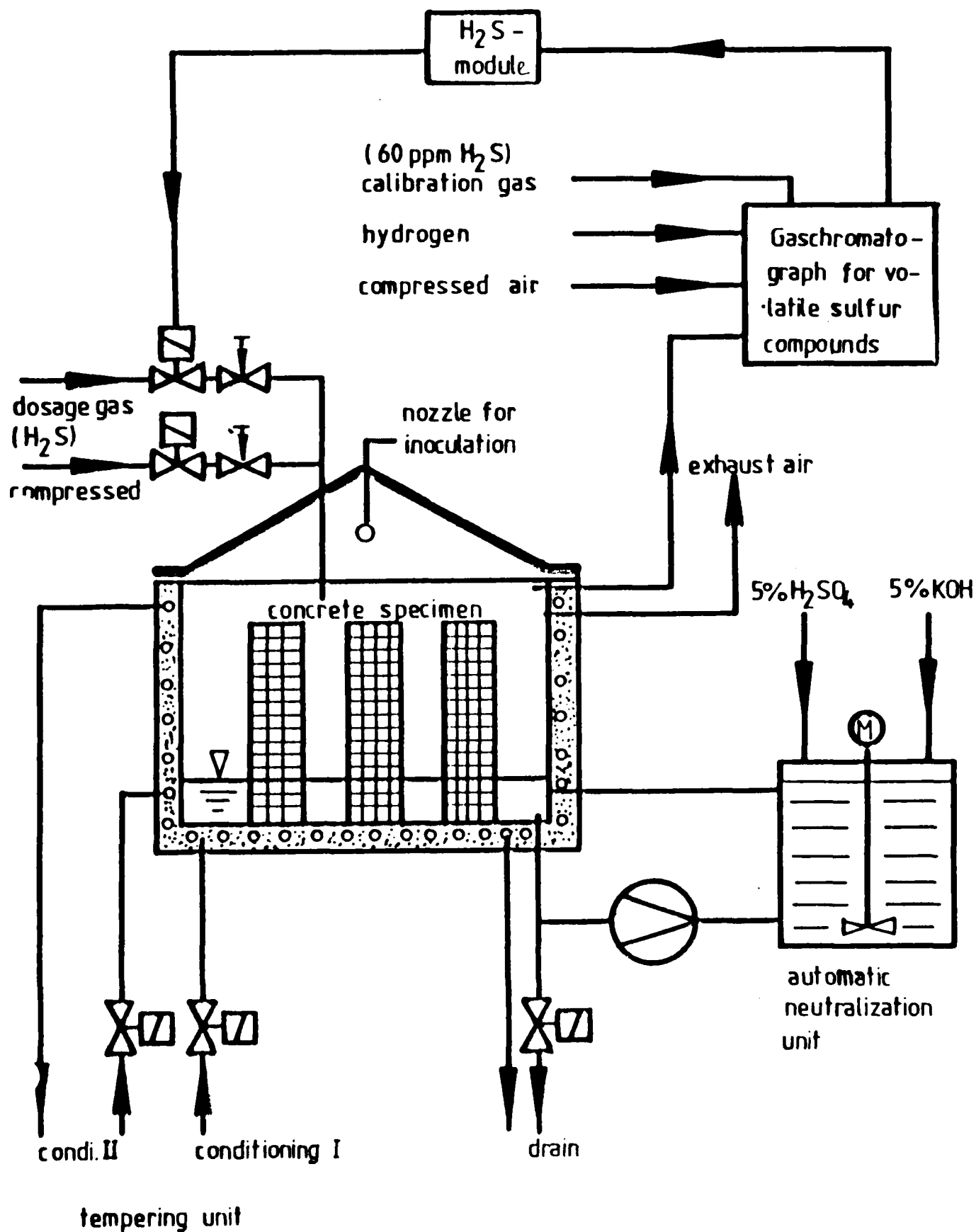
SAND ET AL. FIGURE 2

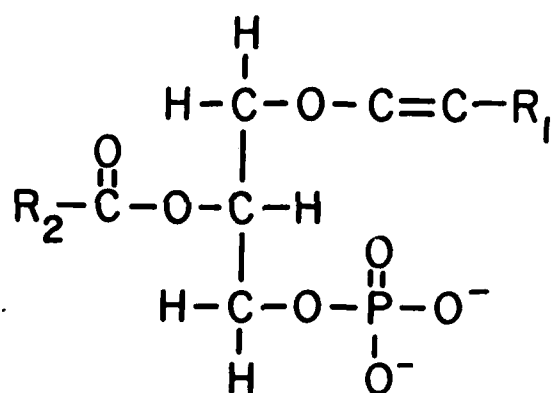Sum et al Fig 3

DIACYL PHOSPHOLIPID
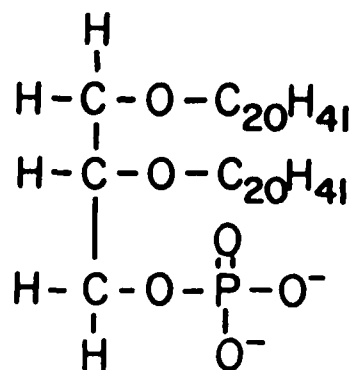
PLASMALOGEN PHOSPHOLIPID

PHYTANYL GLYCEROL ETHER

LIPIDS

DPGE
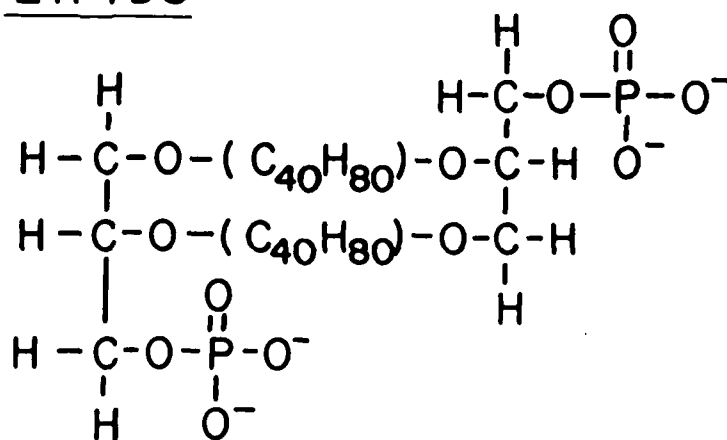
Bi DPGE

# THE ROLE OF CONSORTIA
# IN MICROBIALLY FACILITATED CORROSION

Aerobic seawater

METAL

Microcolony
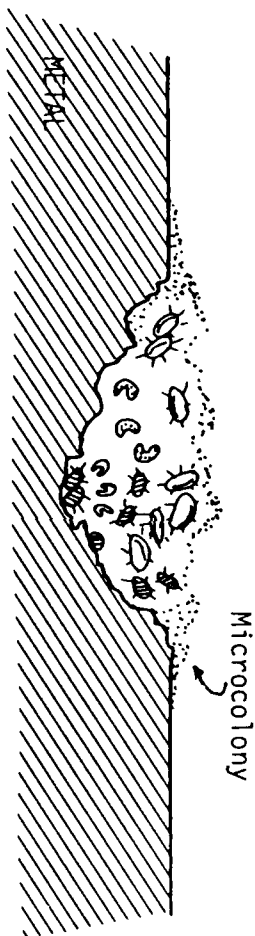
Anaerobic MFC in Aerobic Systems

Heterotrophic Aerobe - creates anaerobic
microniche: Polymer ⟶ Monomer

Acetogen: Monomer ⟶ Acetate

Desulfobacter: $SO_4^-$ + Acetate ⟶ $CO_2$ + $H_2S$

FT/IR - ATTENUATED TOTAL REFLECTANCE

1. Observe living biofilms

ATR crystal
evanescent wave

cell

coating to be
tested

seawater

crystal

ATR crystal surface
evanescent wave

Metal coating
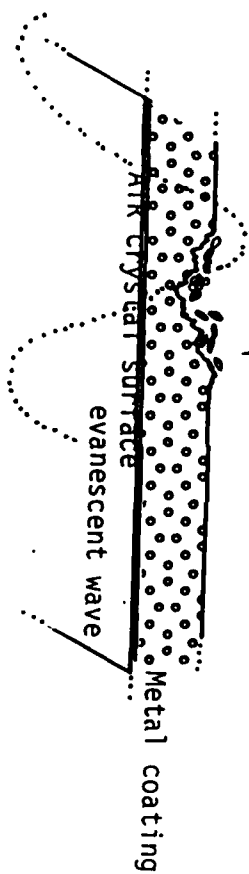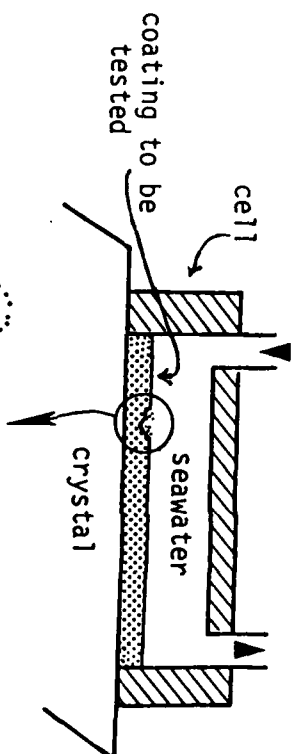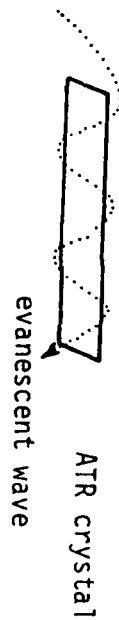
With corrosion see increase in water adsorption
(1640 cm-1)

(Geesey, Mittleman, Iwaoka, Griffiths., Materials Performance,
5:37-40, 1986.)

MECHANISMS OF M.F.C.

1. Metabolic Generation of Acids
   a) anaerobic fermenters

   carbohydrates —————→ Acetic acid
                         Lactic acid
                         etc.

   (Clostridia ————— Plasmalogens)
   (Bacteroides ————— Phosphosphingolipids)

   b) Mineral acid generators

   reduced sulfur —————→ $H_2SO_4$

   ( Thiobacilli  ———  Hydroxy, Cyclopropane)
   ( Thermoplasma ———  Ether lipids)

2. Differential Cathodic Activity

   ///////\\\\\\\        heterogenous distribution

3. Chelation ————→ Shift in Equilibrium
   (Iron Bacteria)

4. Sulfate-reducing bacteria
   Hydrogen removal - (Desulfovibrio~i17:1 7c)
   Sulfide generation - ( Desulfobacter~10Me16:0)

5. Hydrogen Embrittlement
   form atomic hydrogen on surface?

MICROBIOLOGY OF CORROSION

1. The Importance of Exopolymers

   • ADHESION
   • SPECIFIC ASSOCIATIONS
   • PROTECTION - ( Chemical, Mechanical,
                    Biological)
   • CONTROL MICRO-ENVIRONMENT - (Chelation,
                    Deoxygenation)

2. The Formation of Mixed Microbial Consortia

   • Greatly increase metabolic versatility
   • Modify the environment -- microniche
   • Aerobic and anaerobic mechanisms

# ATR Flow Cell Experiments
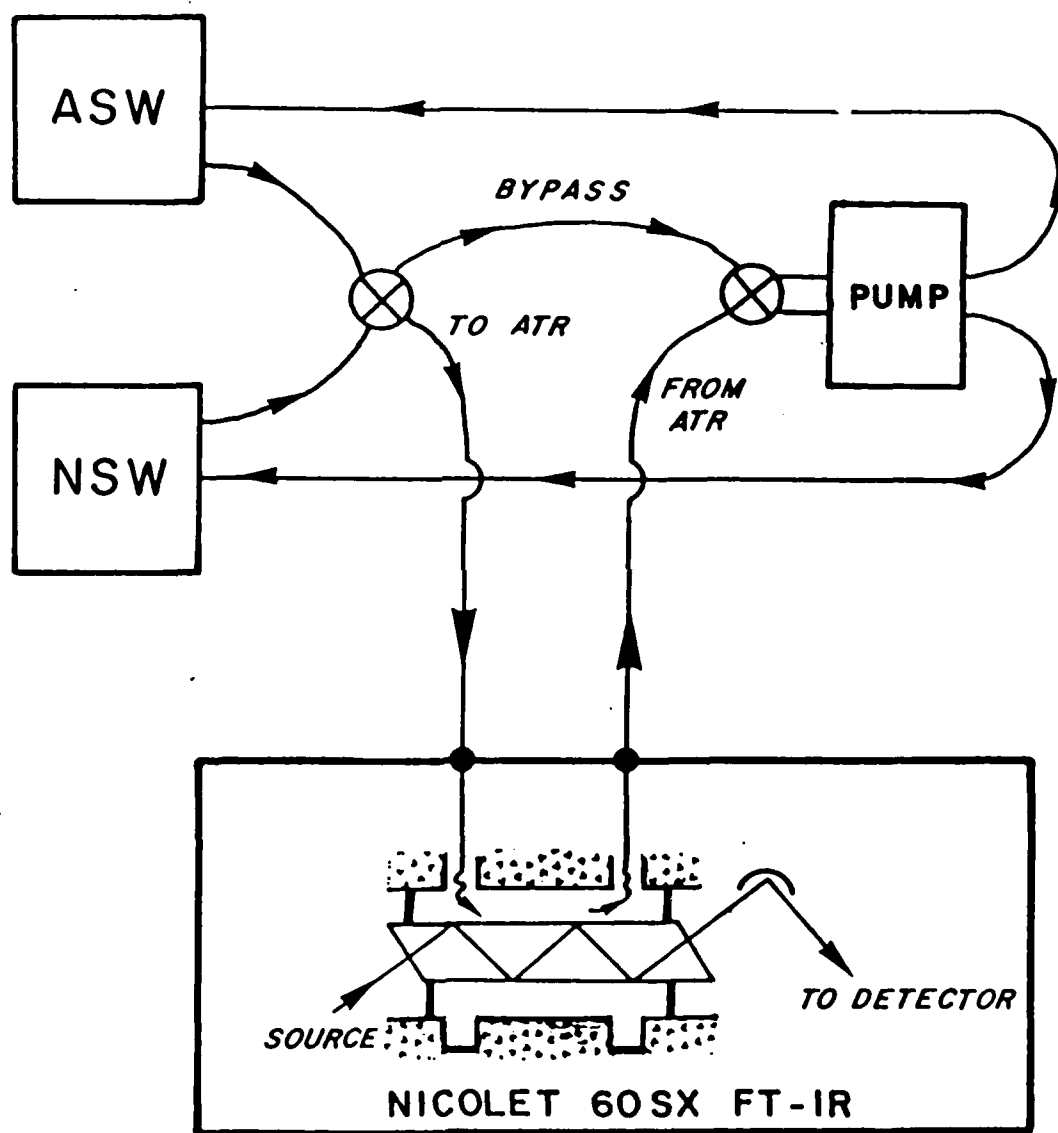
# In situ Real Time Analysis with Attenuated Total Reflectance (ATR)
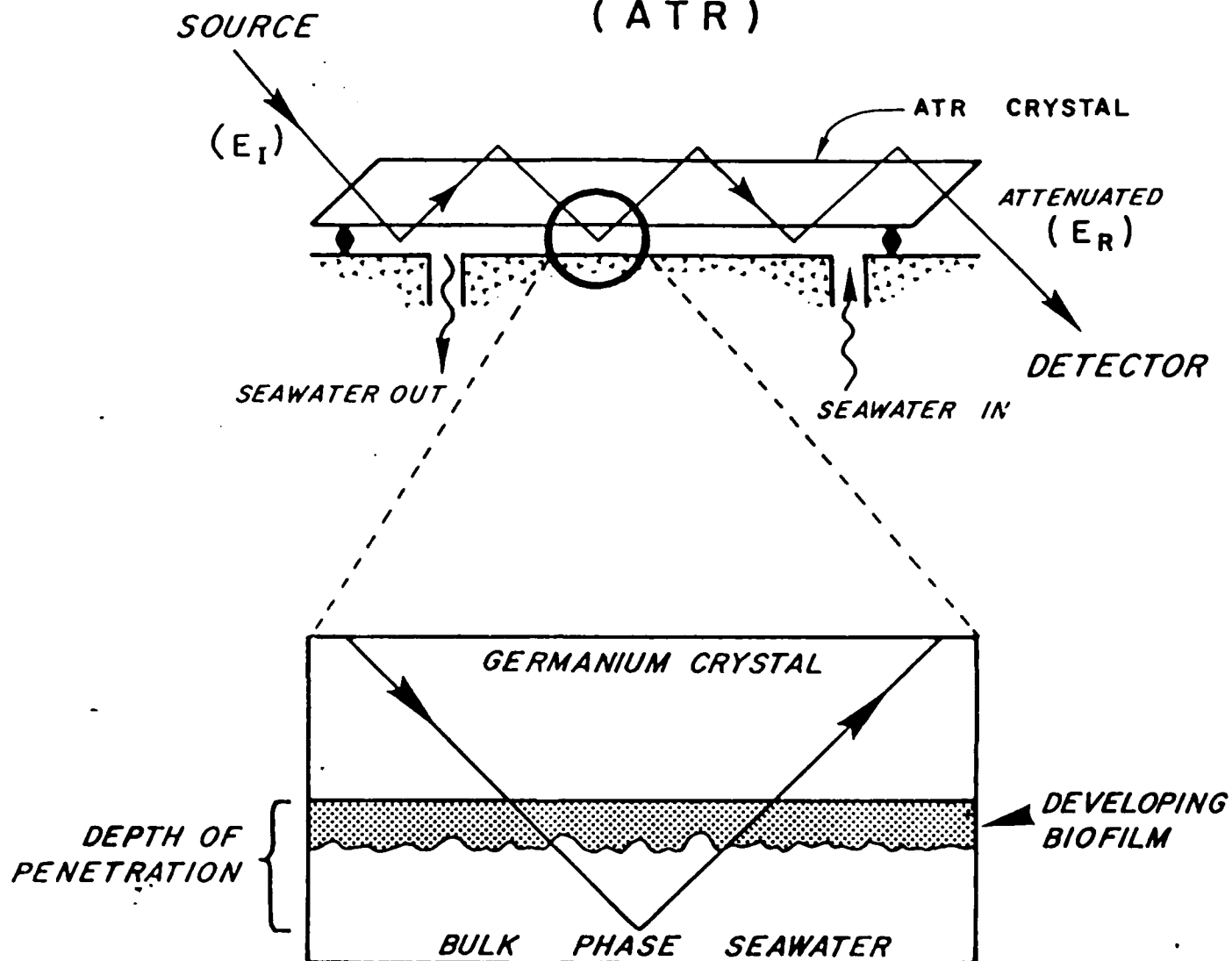
# OBJECTIVES
## "Signatures of Microbial Groups"

1. Fermenters.

Complex CHO $\rightarrow$ $H_2$ + $CO_2$ + short chain acid

$$CH_2-O-CH=CH-R$$
$$CH-O-\overset{O}{\overset{\|}{C}}-R$$
$$CH_2-O-PO_3-X$$
Plasmalogen

$$HO-CH-CH_2-R$$
$$H\overset{|}{C}-NH-\overset{O}{\overset{\|}{C}}-R$$
$$CH_2-O-PO_3-X$$
Sphingolipid
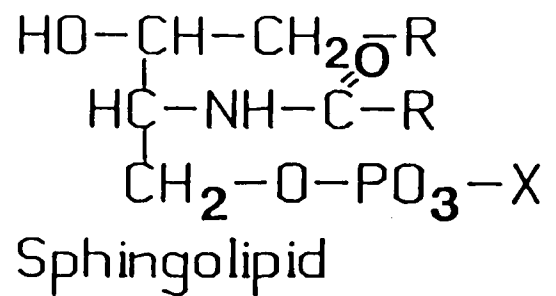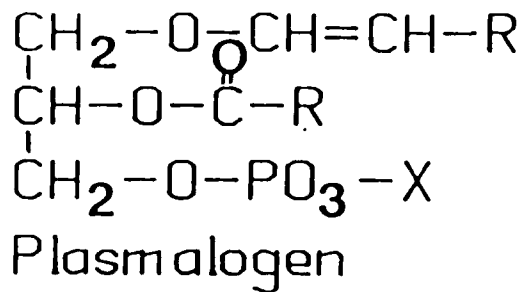
2. Sulfate Reducers.          $H_2$ + $CO_2 \rightarrow H_2S$
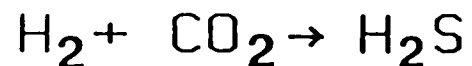
Polar ester−linked fatty acids: Br15:0, i16:0, i17:1w8c, and 10 Methyl 16:0.
Hydroxy Fatty Acids from the LPS.

Methanogens.      $H_2$ + $CO_2 \rightarrow CH_4$
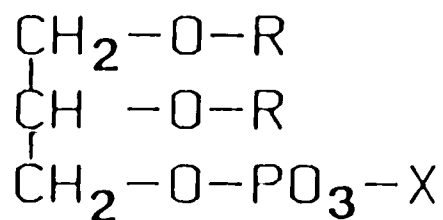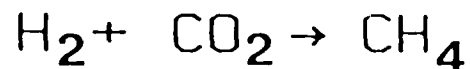
$$CH_2-O-R$$
$$CH-O-R$$
$$CH_2-O-PO_3-X$$
Diphytanyl Glycerol Ethers

## GENERAL PROPERTIES OF CELLS

PHOSPHOLIPID -

1. COMPONENT OF ALL MEMBRANES

2. RAPID TURNOVER

3. STABLE AMOUNTS IN CELLS

    98% OF EUBACTERIAL MEMBRANES

    $\sim$ 50% OF REST

4. QUANTITATIVELY EXTRACTABLE

COUPON

LYOPHILIZATION

SCANNING ELECTRON MICROSCOPY (10)

FT / IR ANALYSIS (9)

ELECTROCHEMICAL MEASUREMENT OF CORROSION CURRENT DENSITY (6)

SOLVENT EXTRACTION (11)

LIPID FRACTIONATION (12)

GLYCOLIPIDS

POLAR LIPIDS

ETHANOLYSIS

GLC ANALYSIS FOR PHA

MILD ALKALINE METHANOLYSIS

LIPID PHOSPHATE FOR BIOMASS (11)

THIN LAYER CHROMATOGRAPHY

FAME

OH - FAME

GLC ANALYSIS (14)

·DIELS - ALDER RXN

GC / MS (15)
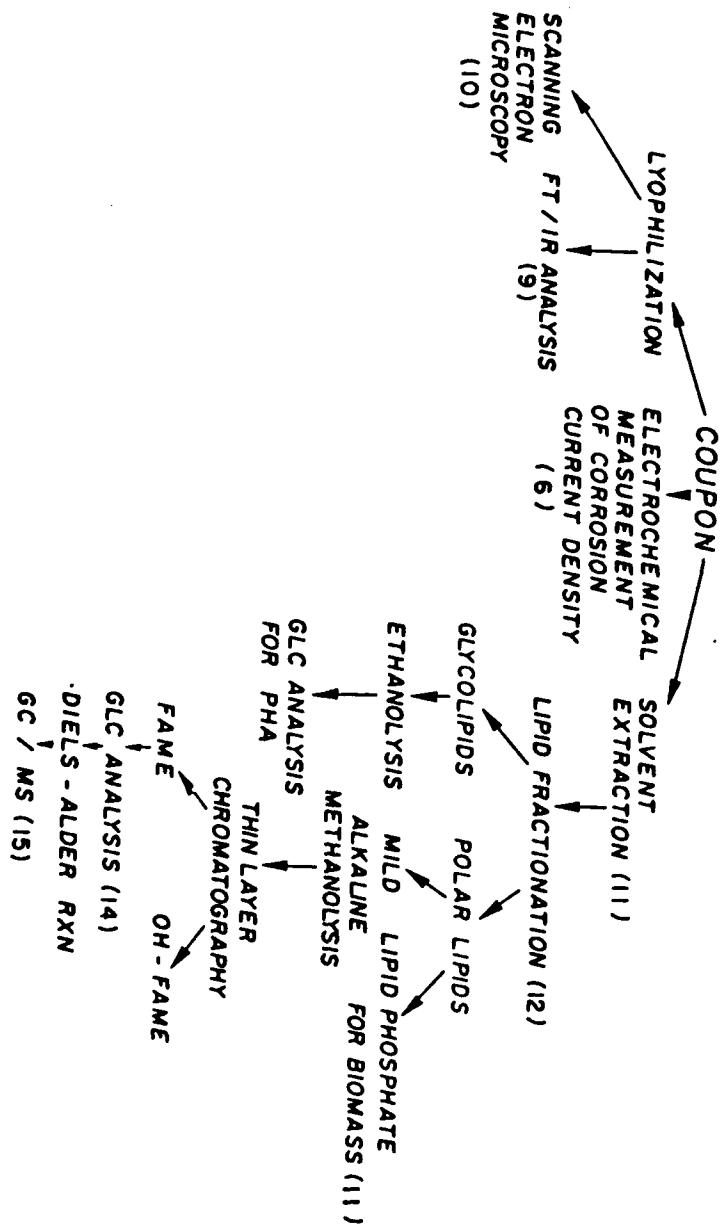
# ASSOCIATION OF ACID PRODUCING THIOBACILLI WITH DEGRADATION OF CONCRETE:

## ANALYSIS BY "SIGNATURE" FATTY ACIDS FROM THE POLAR LIPIDS AND

## LIPOPOLYSACCHARIDE

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* To whom correspondence should be directed

Table 1

## A. ESTER-LINKED FATTY ACID COMPOSITION IN THE POLAR LIPIDS OF THIOBACILLI

NONHYDROXY FATTY ACIDS

Thiobacillus Species

| Fatty Acid | T. intermedius | T. neapolitanus | T. thiooxidans | CHAMBER | SEWER |
|---|---|---|---|---|---|
| | | | mole percent of FAME | | |
| 15:0 | ---- | 3.79 | ---- | 1.36 | 6.84 |
| 16:1w7c + t | 0.87 | 15.1 | 9.19 | 3.25 | 8.54 |
| 16:0 | 15.0 | 28.5 | 36.1 | 15.8 | 23.5 |
| cy17:0w(7,8) | 9.46 | 1.20 | 5.56 | 10.8 | 2.43 |
| 17:0 | 2.64 | 4.61 | <0.3 | 2.46 | 14.5 |
| 18:1w7c + t | 6.63 | 37.2 | 39.5 | 18.8 | 15.9 |
| 18:1w5C | 0.95 | 0.93 | <0.3 | <1.0 | <1.0 |
| 10 + 11Me18:1w6 | 5.97 | 0.41 | 0.70 | <1.0 | <1.0 |
| cy19:0(w8,9) | 25.6 | 1.52 | 4.24 | 32.8 | 14.7 |
| cy20:0 | 11.6 | 0.34 | 1.35 | <1.0 | <1.0 |

AMIDE AND ESTER-LINKED HYDROXY FATTY ACIDS

| OH Fatty Acid | T. intermedius | T. neapolitanus | T. thiooxidans | CHAMBER | SEWER |
|---|---|---|---|---|---|
| | | | mole percent of OHFAME | | |
| 2,3-OH14:0 | 0.59 | 5.95 | 5.74 * | 11.8 * | 21.5 * |
| 2OH-cy16:0 | 6.44 | 28.2 | ---- | 5.99 | 10.7 |
| 2,3-OH16:0 | 53.6 * | 10.9 * | 25.0 * | 16.5 * | 11.1 * |
| 2,3-OH17:0 | 1.28 | 3.32 | 2.92 | 0.96 | 1.73 |
| 2OH-cy18:0 | 11.0 | 38.0 | 1.61 | 3.70 | 1.11 |
| 2, 3OH-18:0 | 5.27 | 5.72 * | 22.6 | 10.3 | 15.2 |
| 2OH-cy19:0 | 15.3 | 8.02 | 42.2 | 27.3 | 6.69 |
| % OHFAME/FAME | 12.3 | 1.5 | 15.3 | 33.1 | 42.4 |

* Indicates found primarily in the polar aminolipid

## B. HYDROXY FATTY ACID COMPOSITION OF THE LIPOPOLYSACCHARIDE OF EXTRACTED THIOBACILLI

| OH Fatty Acid | T. intermedius | T. neapolitanus | T. thiooxidans | CHAMBER | SEWER |
|---|---|---|---|---|---|
| | | | mole percent of OHFAME | | |
| 2,3-OH12:0 | 39.1 | 57.1 | 1.41 | 19.5 | 14.5 |
| 2,3OH-13:0 | 1.98 | 8.94 | 4.37 | <1.0 | <1.0 |
| 2,3OH-14:0 | 6.48 | 5.81 | 20.6 | 26.4 | 21.4 |
| 2,3OH-15:0 | <1.0 | 8.57 | 4.31 | 5.48 | 7.27 |
| 2,3OH-16:0 | 44.2 | 7.93 | 32.6 | 13.3 | 11.6 |
| 2,3OH-17:0 | <1.0 | <1.0 | <1.0 | 1.28 | 2.82 |
| 2,3OH-18:0 | 8.27 | 4.15 | 9.18 | 5.67 | 11.1 |
| 2,3OH-19:0 | <1.0 | 3.41 | 9.82 | 9.62 | 5.42 |

Values in Table 1 represent average values of duplicate analyses.

I

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SUMMARY

The acid-producing thiobacilli contain fatty acid components in the polar lipids and lipolysaccharide lipid A that are sufficiently unusual that they can be utilized as "signature" lipid biomarkers for these organisms in environmental samples. Studies in microcosms have shown correlations between activity of these organisms measured by recovery and viable counting and the degradation of concrete. The "signature" lipid analysis provides a detection assay requiring neither separation of the organisms from the substratum nor growth prior to determination. The presence of acid producing thiobacilli was demonstrated in microcosm samples and degenerating concrete from the Hamburg (FRG) sewer system.


Key words: Concrete biodegradation, Signature lipids, Fatty acids, thiobacilli, Acid producing bacteria, Hydroxy-cyclopropyl fatty acids, Methoxy fatty acids)

## INTRODUCTION

The acid-producing, aerobic, gram-negative thiobacilli have been shown to produce a diverse complement of fatty acids in the chloroform:methanol extractable phospholipids [4]. The combination of o-methoxy, mid-chain hydroxy, 2-hydroxy cyclopropane, midchain-branched, branched-cyclopropane, monounsaturated at the omega (w) 5, 6, 7, 8, 9, 10 positions, 2- and 3- hydroxy, and large proportions of the "thiobacillic" cyclopropane 19:0 w(8,9) fatty acids are found in the polar lipids of these organisms. These polar lipid fatty acids (PLFA) are sufficiently unique among the microbes that they can serve as effective "signatures" for these organisms.

Failure of concrete sewers has been associated with acid-producing microorganisms that utilize reduced sulfur and oxygen with the generation of sulfuric acid [9, 14, 17, 19]. To study the resistance of different concrete samples to the effects of the thiobacilli, Sand and Bock constructed a chamber in which the temperature, humidity, and hydrogen sulfide concentration could be regulated [16, 17] and were able to greatly accelerate the biodegradation. In the field and in the artificial chamber the most rapid biodegradation was associated with the growth of Thiobacillus thiooxidans [9, 16]. The detection of the thiobacilli in these studies involves the isolation and culture on several media. This can require six to eight weeks. The present study will show that the presence of the acid producing thiobacilli can be detected in samples from the Hamburg sewer and from the biotest chamber utilizing the "signature" biomarkers from the extractable PLFA, and the lipopolysaccharide lipid A (LPS). Signature lipid biomarkers been utilized to detect type I and II methanotrophic bacteria [11, 13], Desulfobacter and Desulfovibrio sulfate-reducing bacteria

[1, 2], the pathogenic bacterium <u>Francisella</u> <u>tularensis</u> [10], and the archaebacterial methanogenic archaebacteria [7, 8].

## MATERIALS AND METHODS

### Materials

Solvents and reagents were the best grade available commercially. Standards and derivatizing agents were purchased from Supelco, Inc. (Bellefonte, PA), Applied Science (State College, PA), Aldrich, Inc. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), and Pierce Chemical Co. (Rockford, IL).

### Samples

<u>Thiobacillus</u> <u>thiooxidans</u> strain K-2, <u>T.</u> <u>neapolitanus</u> strain B-2, and <u>T. intermedius</u> strain D-14 were grown and recovered in the late stationary phase by centrifugation as described previously [4, 16]. Concrete samples from the sewer or biotest chamber were lyophilized and then pulverized to chunks smaller than 1 $cm^3$.

### Extraction

The modified one-phase Bligh and Dyer extraction was utilized for all samples [22]. Duplicate samples were extracted separately and all data is expressed as the mean of two determinations. After the overnight separation of the lipid and aqueous phases in the second stage of the extraction, the organic fraction was filtered through fluted Whatman 2V filters and evaporated to dryness under a stream of nitrogen. To recover the polar lipids, silicic acid columns were prepared using 1 g Unisil (100-200 mesh), (Clarkson Chemical Co., Inc, Williamsport, PA) activated at 120°C for 60 min and pre-extracted with chloroform. The columns were 14-mm diameter glass columns. Total lipid was applied to the top of the columns in a minimal volume of chloroform. Sequential washes of 10 ml of chloroform, acetone, and

methanol eluted the neutral, glyco-, and polar lipids respectively. The polar lipid fraction was dried under a stream of nitrogen. The mild alkaline methanolysis procedure [22] was utilized to prepare methyl esters of the ester-linked PLFA.

### Lipopolysaccharide analysis

The residue from the lipid extraction of the cells was refluxed in 3 N HCl for 4 hours and the lipid fraction recovered in chloroform [15]. The concrete samples were refluxed in 5% (w/v) KOH in methanol:water (8:2) for 4 hours, allowed to cool, the hydrolysate separated from the concrete by repeated washes with water and chloroform, which was neutralized with HCl to pH 6.0. The solution was partitioned against chloroform and the lipid soluble hydrolysate recovered.

### Purification of the lipids

Thin layer chromatography (TLC) on Whatman K-6 silica gel, 0.250 mm thick was utilized for purification. The methyl esters from the polar lipid fraction were applied in a strip to the origin of the TLC plate that had previously been divided into a large mid plate channel with two narrow channels on the edges. Authentic fatty acid methyl esters (FAME) and hydroxy fatty acid methyl esters (OHFAME) were applied to the outside channels and the plate placed in a tank for separation by ascending chromatography in a solvent of chloroform: methanol: water (55: 35: 6, v/v/v). The silica gel bands corresponding to the FAME and OHFAME ($R_f$ >0.8) were lifted from the plate with vacuum in a Pasteur pipette and the fatty acids recovered with chloroform: methanol (2:1, v/v). The position of the aminolipids was identified by spraying a portion of the plate with 0.25% w/v ninhydrin in acetone: lutidine (9: 1, v/v) [4]. The bands on the unsprayed portion of the plate were recovered in a Pasteur column and eluted with chloroform: methanol (1: 1) and (2: 1, v/v).

The FAME and OHFAME were separated using ascending chromatography with hexane: diethyl ether (1: 1, v/v). The FAME band ($R_f$ 0.65) and OHFAME band ($R_f$ 0.25) were recovered and eluted with chloroform and chloroform: methanol (1: 1, v/v).

The aminolipids recovered from the TLC plates were subjected to acid methanolysis in anhydrous methanol: concentrated HCl: chloroform (10: 1: 1, v/v/v) after heating at $100^{\circ}$C for 1 h. The OHFAME were recovered in chloroform.

### Derivatizations

Trimethylsilyl ethers of OHFAME were formed with N, O,-bis-(trimethylsilyl)-trifluoroacetamide (BSFTA) (Pierce Chemical Co., Rockford, IL) [15].

The position and geometry of the monounsaturation in the FAME and OHFAME was determined using two procedures. Dimethyl disulfide (DMDS) adducts were prepared as described [12]. These derivatives increase the resolution between cis and trans geometrical isomers in capillary gas-liquid chromatography (GC). The position of the cyclopropane ring in the FAME can be determined after hydrogenation in the presence of Adam's catalyst of $PtO_2$ with the esters dissolved in methanol:glacial acetic acid, (1: 1, v/v) under a hydrogen atmosphere (140 kPa) at room temperature with mechanical agitation for 20-40 h in a Parr hydrogenation apparatus (Moline, IL) [4]. These derivatives give fragmentation patterns at branch points on either side of the original cyclopropane ring. Similar treatment of the 2-OHcy FAME did not yield fragments allowing determination of the branch points.

### Gas chromatography (GC)

Dry FAME or OHFAME were dissolved in hexane and the internal standard of methyl nonadecanoate added. Samples of 1.0 ul were injected onto a 50-m

nonpolar, cross-linked methyl silicone fused silica capillary column (0.2-mm i.d., Hewlett Packard) in a Varian 3700 GC. A 30-s splitless injection with the injection temperature at 250°C was used. Hydrogen at a linear velocity of 35 cm/s was the carrier gas with a temperature program starting with an initial temperature of 80°C. The initial 20°C/min rise for 3 min followed by a 4°C/min rise for 30 min and an isothermal period for the remainder of the 40-min program was utililzed. Detection was by hydrogen flame (F. I. D.) using a 30 ml/min nitrogen make up gas at a temperature of 290°C. An equal detector response was assumed for all components. Peak areas were quantified with a programmable laboratory data system (Hewlett Packard 3350 series) operated in an internal standard program relative to known amounts of internal standard.

### Gas chromatography/mass spectrometry (GC/MS)

FAME and OHFAME were tentatively identified by co-elution with authentic standards supplied by Supelco, Inc. (Bellefonte, PA) and Applied Science Labs., Inc. (State College. PA) or previously identified laboratory standards. The analysis was performed on a Hewlett Packard 5996A GC/MS with a direct capillary inlet utilizing the same chromatographic system except for the temperature program which was begun at 100°C and increased to 280°C at 4°. C/min for a total analysis time of 60 min. The electron multiplier voltage was between 1400 and 1600 V, the transfer line maintained at 300°C, the source and analyzer maintained at 250°C, and the GC/MS was autotuned with DFTPP (decafluorotriphenylphosphine) at m/z 502 with an ionization energy of 70 eV. The data was acquired and manipulated using the Hewlett Packard RTE 6/VM data system. Other conditions were as described previously [4].

### Fatty acid nomenclature

Fatty acids are designated as total number of carbon atoms: number of double bonds with the position of the double bond nearest to the aliphatic

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(w) end of the molecule indicated. This is followed by the suffix c for cis and t for trans configuration of monoenoic fatty acids. The prefixes i, a, or br indicate iso, anteiso, or branched (position undetermined). Mid chain branching is indicated by the number of carbon atoms from the carboxyl ($\triangle$) end of the molecule and Me for the methyl group. Cyclopropane rings are indicated with the prefix cy and the position of the ring from the aliphatic (w) end of the molecule. Hydroxy fatty acids are indicated by the number of carbon atoms from the carboxyl end of the molecule followed by the prefix OH. Methoxy fatty acids are given with the number of carbon atoms from the carboxyl end of the molecule followed by the prefix MeO.

## RESULTS

### Detection of thiobacilli

The proportions of ester-linked FAME and both ester- and amide-linked OHFAME in the polar lipids in the thiobacilli isolated from corroding concrete, as test concrete blocks exposed in a biotest chamber to thiobacilli, and a sample from a corroding concrete sewer pipe, are listed in Table 1,A. The OHFAME from the LPS-lipid A of the lipid extracted residue of the isolated monocultures and the test and environmental samples are given in Table 1, B. The similarity in the patterns of proportions of FAME and OHFAME from the monocultures, biotest samples, and corroded sewer sample indicate that the acid producing thiobacilli are a prominent part of the microflora in both the biotest and sewer samples. The low proportions of the usual bacterial PLFA such as 16:0, 18:1w9c, the elevated proportions of cy17:0w(7, 8) and especially the "thiobacillic" acid cy19:0w(7,8) in the extractable polar lipid FAME, the 2, and 3-OH16:0 in the ornithine-lipid, and the high proportion of OHFAME in the PLFA as well as the high proportion of 2, and 3-OH16:0 in the LPS lipid A are typical of the acid producing thiobacilli. Hydroxy cyclopropane fatty acids found in both the extractable polar lipids and the LPS lipid A are typical of the acid producing thiobacilli (4).

### Detection of T. thiooxidans

The elevated proportions of 2-OH14:0, 2-OH16:0, and particularly 1-OHcy19:0, the high OHFAME/FAME ratio in the extractable polar lipids of the biotest chamber and environmental samples together with the high proportions of 3-OH14:0 and 2, 3-OH19:0 suggest that T. thiooxidans is a predominant member of the microbial consortia in the environmental samples. The higher proportions of 3-OH12:0 in both the biotest sample and the environmental samples and T. intermedius and T. neapolitanus than in the T. thiooxidans monoculture indicate that the other thiobacilli are also part of the community.

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# DISCUSSION

## Signature PLFA

It has been possible to utilize the signature lipid biomarker technique to detect specific microorganisms in complex environmental samples. Methane oxidizing auxotrophs contain PLFA that are sufficiently unusual that they can be utilized as biomarkers [11]. Exposure of columns of sub-surface soil to natural gas results in a great increase in the specific PLFA of type II methylotrophs [13]. The methanogenic archaebacteria contain bi- and bidiphytanyl glycerol ether polar lipids which can be assayed in environmental samples and correlated with methanogenic activity in soils and sediments [7, 8]. The pathogenic bacteria Francisella tularensis and the sulfate-reducing bacterial groups of lactate-utilizing Desulfovibrio and acetate-utilizing Desulfobacter have been shown to contain sufficiently unusual patterns of PLFA to allow their assessment in mixed microbial assemblies [1, 2, 10]. The incredible diversity of unusual PLFA in the acid producing thiobacilli allows their detection in environmental samples (Table 1). (With samples to allow replication the species differences detected in monocultures [4, Table 1] can give insight into the community structure of these organisms as well as into the other physiological groups of organisms present [6, 9].) Patterns of PLFA which to define the community structure of microbial consortia have been utilized to show detrital succession, the effects disturbance or predation upon marine sediments, the response to subsurface aquifer pollution, in environmental effects testing, and by the effects of shifts in the microbiota biofouling succession and facilitation of corrosion [18, 19-21].

## Metabolic status

Many bacteria accumulate cyclopropane fatty acids as the community ages or undergoes nutritional stress. Their formation with the concomitant decrease in monoenoic PLFA occurs in monocultures that undergo metabolic

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stress such as stationary phase growth [3, 5]. This same phenomenon has
been detected in the benthic marine microbiota [3, 21] as well as the acid
producing thiobacilli [4]. The acid producing thiobacilli from the
chamber samples or from the degenerating concrete sewers both show the high
levels of cyclopropane *PFA* and low levels of monoenoic PLFA characteristic of
late stationary growth phase.

### Role of acid producing thiobacilli in biodegradations

The correlation between the degree of biodeterioration of concrete and the
activity of acid producing thiobacilli has been shown using classical
recovery and culture techniques [9, 16, 17]. The studies reported in this
paper indicate that the PLFA patterns are sufficiently unique to define the
presence of the acid producing thiobacilli and possibly define the particular
species that are present without the uncertainties and time delays of
cultural methods. In addition the PLFA offer insights into the community
structure and metabolic status of the total microbial community associated
with the biodegradations of materials that involve these organisms.
Preliminary evidence from both corroding sewer systems and from a continuous
culture apparatus designed to test the resistance of concrete samples to the
corrosive activities of acid producing bacteria shows that the degree of
biodegradation appears to correlate with the presence of "signature" PLFA of
the acid producing thiobacilli, particularly T. thiooxidans [9, 16, 17]. The
methodology described herein will allow examination of an entire microbial
ecosystem so the interactions between the acid producing thiobacilli and the
other organisms that potentiate their corrosive activities can be defined
[6].

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Detection of a Microbial Consortium Including Type II Methanotrophs

by Use of Phospholipid Fatty Acids in an Aerobic Halogenated

Hydrocarbon-degrading Soil Column Enriched with Natural Gas

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ABSTRACT

The phospholipid ester-linked normal and lipopolysaccharide hydroxy fatty acids from microbes in a halogenated hydrocarbon-degrading (HHD) soil column have been analyzed in detail with capillary gas chromatography-mass spectrometry (GC-MS). An increase in microbial biomass was observed when the HHD column was compared to a control azide-inhibited column and to uncontaminated surface soil. Biomass estimates for the upper 10 cm of the HHD column averaged $5.6 \times 10^9$ cells/g (dry weight) of sediment based on phospholipid fatty acids (PLFA). Microbial community structure information was obtained following GC-MS analysis of derivatized monounsaturated PLFA. The major component (16-28% of the PLFA) detected in the HHD soil column was 18:1Δ10c. This relatively novel fatty acid has been previously reported as a major component in methanotrophs and its presence in the soil is consistent with a large contribution of this microbial metabolic group to the column flora. The high relative proportions of $C_{18}$ components relative to $C_{16}$ fatty acids indicates that type II rather than type I methanotrophs are the most abundant microbial flora present. Other microbial groups recognized in the HHD soil column, in decreasing order of abundance, were Actinomycetes, the sulphate-reducing bacteria Desulfovibrio spp., and microeukaryotes. Differences between the relative proportions of these metabolic groups of microorganisms have been quantified and contrasted between the soils analyzed. Based on these differences, the potential exists to use these methods for monitoring shifts in microbial biomass and community structure in aquifers in which similar indigenous bacteria are stimulated to biotransform pollutant substrates.

## INTRODUCTION

Bacteria capable of growth with methane as the sole carbon and energy source are known as methanotrophs (1, 17). The physiology and ecology of methanotrophic bacteria are described in recent reviews (1, 6, 16, 17, 18, 20, 29, 34). Methane monooxygenase (MMO) is used by these bacteria to oxidize methane to methanol (1, 16, 17). MMO is also capable of producing primary or secondary alcohols upon oxidation of alkanes up to octane (7, 30) and, in addition, can oxidize halogenated one-carbon compounds (7). Halogenated one- and two-carbon compounds are commonly detected in contaminated subsurface environments and ground water (30). Wilson and Wilson (42) used natural gas to enrich a population of bacteria in soil capable of degrading trichloroethylene (TCE). TCE was degraded to carbon dioxide in the presence of natural gas (77% methane, 10% ethane, 7% propane, remainder contained 4 to 7 hydrocarbons; 42). It appears, therefore, that methanotrophic bacteria may be useful in the removal of halogenated one- or two-carbon compounds from contaminated environments (unpublished data). An estimate of their biomass in natural systems would thus be useful in optimizing conditions for their growth and activity. Prior to this study, methods for the direct measurements of methanotrophic bacteria in microcosms or field samples did not exist.

Methanotrophic bacteria are grouped into two divisions, types I and II, based on differences in intracytoplasmic membrane organization and carbon metabolism (1, 17). Analysis of cellular fatty acid profiles has become a standard tool in chemotaxonomy (14). Type I methanotrophic bacteria contain esterified fatty acids, predominantly 16 carbons in length, with saturated (16:0) and monounsaturated (16:1) fatty acids present (22, 34). Type II methanotrophic bacteria have monounsaturated

18-carbon chain-length (18:1) fatty acids as the predominant PLFA (22, 34).
Fatty acids are used as biomarkers or signature lipids in microbial ecology
and often provide valuable information on the structure of the microbial
community (38). If membrane fatty acids are to be used as biomarkers by
taxonomists, ecologists, and geochemists, precise determination of double-
bond positions and geometry will be essential for correct interpretation of
increasingly complex data sets. A number of relatively simple and rapid
procedures have recently been reported that allow such determinations to be
routinely performed (eg. 8). The difference in carbon chain length and,
more importantly, position and geometry of unsaturation (22, 24) suggest
that the results of analysis of extractable phospholipid fatty acids (PLFA)
from an ecosystem would indicate the presence or absence of methanotrophic
bacteria. Similarly, PLFA profiles may be useful for characterizing other
microbial groups, capable of degrading short-chain hydrocarbon, present in
soils.

The phospholipid ester-linked and lipopolysaccharide(LPS) normal and
hydroxy fatty acid profiles of sediment from a manipulated halogenated
hydrocarbon-degrading (HHD) soil column are reported here. The overall aim
of this study is to identify specific lipid components that can be used to
monitor for methanotrophic bacteria. These lipid biomarkers can be used
for interpretation not only of the manipulated laboratory microcosms
analyzed here but also of samples taken from field experiments.

## MATERIALS AND METHODS

### Soil and column description

The column was essentially as previously described (40, 42). The soil
sampled was taken from Lincoln fine sand (a mixed, thermic typic
Ustifluvent) at a site near Ada, OK. The soil was collected in 10-cm
increments, returned rapidly to the laboratory, and packed into glass
columns 5 cm I.D. x 150 cm before it could lose significant moisture. Each
10-cm increment was packed in the same relative position it occupied in the
original soil profile. The average bulk density of the columns was 1.65
$g/cm^3$. The saturated hydrolic consistency ranged from 120 to 190 cm/day.
The columns received 21 $cm^3$/day of tap water from Ada, Ok., amended with
the suite of organic compounds decribed below. One column (A) was exposed
to a headspace of 0.6% natural gas in air and received the following
halogenated hydrocarbons, concentrations in µg/l: carbon tetrachloride,
1100; chloroform, 210; dichloromethane, 270; tetrachloroethylene, 700; tri-
chloroethylene, 1000; cis- and trans-1,2-dichloroethylene, 170, 190;
1,1,1-trichloroethane, 210; 1,1,2-trichloroethane, 290; 1,1-dichloroethane,
240; 1,2-dichloroethane, 280; and 1,2-dibromoethane (ethylene dibromide),
370. Only trichloroethylene was studied in a previous report on the soil
column exposed to methane (42). Complete results of the degradation of
these compounds will be reported separately (J. M. Henson, J. W. Cochran,
and J. T. Wilson, manuscript submitted). Approximately 50% or less of
carbon tetrachloride, tetrachloroethylene, 1,1,1-trichloroethane, and
1,1,2-trichloroethane were microbially transformed, and greater than 75% of
each of the other compounds were similarly transformed. The second column
(B) was inhibited by addition of 0.1% sodium azide to the water. The

poisoned column was not exposed to natural gas. After three months of operation at 24-29°C, the columns were unpacked, and increments were lyophilized prior to lipid extraction. Control samples (0-10 cm and 148-150 cm) were acquired at a later date (in early summer rather than late fall) from the same site and were lyophilized as above. For samples from both depths, pH ranged from 6.6 to 6.1, cation exchange capacity from 4.8 to 2.3 meq/100 gms, sand from 95 to 89%, silt from 8.8 to 4.0%, and clay from 3.5 to 1.5%. Organic carbon contents of the 0-10 cm and 140-150 cm were 0.20 to 0.22% and 0.02% respectively.

## Lipid extraction and fractionation

Soil samples were placed in a 250-ml separatory funnel, and the lipids were quantitatively extracted with the modified one-phase chloroform methanol Bligh and Dyer extraction (39). After separation of phases, the lipids were recovered in the chloroform layer, the solvents removed in vacuo, and the lipids stored under nitrogen at -20°C. Duplicate extractions of each sediment sample were performed.

The lipid was transferred in a minimum volume of chloroform to a silicic acid column (Unisil, 100-200 mesh, Clarkson Chemical Co., Inc., Williamsport, PA) and fractionated into neutral lipids, glycolipids, and phospholipids by elution with chloroform, acetone, and methanol respectively (11, 15). The fractions were collected in test tubes fitted with Teflon-lined, screw-cap lids and dried under a stream of nitrogen. The mild alkaline methanolysis procedure (39) was applied to the phospholipid fraction. The technique was modified slightly in that hexane:chloroform (4:1, v:v), rather than chloroform, was used to extract the resulting fatty acid methyl esters (FAME). LPS normal and hydroxy fatty acids were recovered by acidification of the sediment residue in 50 ml of 1N HCl. After being

refluxed at 100°C for 3 h and cooled, the contents were transferred to a separatory funnel with washes of 25 ml and 2 x 5 ml of chloroform. The two phases were allowed to separate overnight, the chloroform phase recovered, and the solvent removed under a stream of nitrogen. The LPS fatty acids were then methylated and converted to their corresponding trimethylsilyl (TMSi) ethers with N,O-bis-(trimethylsilyl) trifluoroacetamide (Pierce Chemical Co., Rockford, IL) (24).

## Gas chromatography

FAME samples were taken up in hexane with methylnonadecanoate (19:0) as the internal injection standard. Separation of individual normal and hydroxy fatty acids was performed by high resolution gas chromatography using a Hewlett Packard 5880A gas chromatograph equipped with a flame ionization detector. Samples were injected at 50°C in the splitless mode with a Hewlett Packard 7672 automatic sampler onto a non-polar cross-linked methyl silicone capillary column (50 m x 0.2 mm i.d., Hewlett Packard). The oven was temperature programmed from 50 to 160°C at 10°C per minute, then at 2°C per minute to 300°C. Hydrogen was used as the carrier gas (1 ml/minute). The injector and detector were maintained at 300°C.

Tentative peak identification, prior to GC-MS analysis, was based on comparison of retention times with those obtained for standards from Supelco Inc. (Bellefonte, PA) and Applied Science Laboratories Inc. (State College, PA) and previously identified laboratory standards. Peak areas were quantified with a Hewlett Packard 3350 series programmable laboratory data system operated in an internal standard program. Fatty acid compositional data reported for these samples are the means of two analyses each. Standard deviations for individual fatty acids were generally in the range 0-30%, typically <10%.

## Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed on a Hewlett Packard 5996A system fitted with a direct capillary inlet. The same column type described above was used for analyses. Samples were injected in the splitless mode at 100°C with a 0.5-minute venting time, after which the oven temperature was programmed to 300°C at either 3 or 4°C per minute. Helium was used as the carrier gas. MS operating parameters were: electron multiplier between 1300 and 1400 volts, transfer line 300°C, source and analyser 250°C, autotune file DFTPP normalized, optics tuned at $m/z$ 502, electron impact energy 70 eV. Mass spectral data were acquired and processed with a Hewlett Packard RTE-6/VM data system.

## Determination of fatty acid double-bond configuration

The dimethyldisulfide (DMDS) adducts of monounsaturated FAME were formed, by the method described by Dunkelblum et al. (8), to locate the double-bond positions. Samples in hexane (50 µl) were treated with 100 µl DMDS (gold label, Aldrich Chemical Co. Milwaukee, WI) and 1-2 drops of iodine solution (6.0% w:v in diethyl ether). The reaction took place in a standard GC vial (Varian Pty. Ltd., Sunnyvale, CA) fitted with a teflon-lined screw-cap lid. After reaction at 50°C in a GC oven for 48 hours, the mixture was cooled and diluted with hexane (200 µl). Iodine was removed by shaking with 5% (w:v) aqueous $Na_2S_2O_3$ (100 µl). The organic layer was removed, and the aqueous layer reextracted with hexane:chloroform (4:1, v:v). The combined organic layers were concentrated under a stream of nitrogen prior to subsequent GC analysis. GC-MS analysis of the DMDS adducts showed major ions attributable to fragmentation between the $CH_3S$ groups at the original site of unsaturation. Discrimination between cis and trans geometry in the original monounsaturated FAME is possible. The

erythro isomer (originally the trans fatty acid) elutes after the threo isomer (originally the cis fatty acid). The different positional isomers of the same geometry were chromatographically separated under the conditions used in this study.

## Fatty acid nomenclature

Fatty acids are designated by total number of carbon atoms : number of double bonds, followed by the position of the double bond from the Δ(carboxylic) end of the molecule. The suffixes c and t indicate cis and trans geometry. The prefixes i and a refer to iso and anteiso branching respectively, and the prefix OH indicates a hydroxy group at the position indicated. Other methyl-branching is indicated as position of the additional methyl carbon from the carboxylic acid (Δ) end, i.e. 10 methyl 16:0. Cyclopropane fatty acids are designated with the prefix cy, with the ring position relative to the carboxylic end of the molecule in parentheses.

## RESULTS

### Phospholipid ester-linked fatty acid (PLFA) biomass estimates

The PLFA concentration data (Table 1) was converted into number of cells per gram of sediment by means of the following approximations (39): $5.9 \times 10^{12}$ bacteria/g (dry weight of cells) with an average methanotroph containing 57 $\mu$moles PLFA/g (dry weight; 24). Biomass estimates of $5.6 \times 10^9$ (range $4.1 \times 10^9 - 7.7 \times 10^9$), $9.3 \times 10^8$, and $2.4 \times 10^9$ cells/g (dry weight) were determined for the upper 10 cm of the methane enrichment column (column A), the azide-inhibited column (column B), and the untreated control surface soil respectively (Table 1). Sediment taken from the bottom (148-150 cm) of the two columns contained at least two orders of magnitude less biomass than the upper layers in each column.

### Fatty acid profiles

A total of 40 normal phospholipid ester-linked fatty acids were positively identified in the columns and soil samples (Table 2). Sixteen monounsaturated components were present, and characteristic ion fragments of the derivatized products formed by reaction with DMDS are shown in Table 3. Interpretation of these data provided the primary information for assignment of double-bond position and geometry.

A number of features were apparent when the fatty acid profiles were compared (Table 2): (i) Several relatively novel monounsaturated fatty acids, 16:1Δ8c, 16:1Δ10c, and 18:1Δ10c, were present in the methane-enriched HHD samples (column A), but absent in column B and the surface soil sample. The latter fatty acid was the major component in all methane-exposed column A samples from 2-10 cm and showed an increase with depth relative to the more common bacterial component 18:1Δ11c (28) (Figure 1). (ii) A series of

10 methyl branched fatty acids was detected in all 0-10 cm samples. A higher relative abundance of these components occurred in column B and control soil than in soil from column A. (iii) The combined relative levels of 16:0 and 16:1 isomers and 18:0 and 18:1 isomers showed minor changes within the upper 10 cm of column A. The sum of the $C_{18}$ components was generally 1.5 to 2 times that of the $C_{16}$ fatty acids in these samples (Figure 2). In contrast, both the surface soil and azide-inhibited control column (B) contained higher relative proportions of the $C_{16}$ fatty acids. (iv) $C_{18}$ and $C_{20}$ polyunsaturated fatty acids (PUFA) were detected in all samples from the upper 10 cm. The untreated surface sediment control contained the highest relative level of 18:2$\Delta$9, whereas the $C_{20}$ PUFA, 20:4$\Delta$5 and 20:5$\Delta$5, were present at similar relative levels in column A and the surface sediment. The latter two components were not detected in soil from the bottom of column A and were at a reduced relative level in column B soil. (v) Cyclopropyl fatty acids, cy17:0 and cy19:0, were detected in all samples, with the highest relative proportions present in the nonhalo-genated hydrocarbon-degrading samples. (vi) A series of saturated FAME, tentatively identified as dimethyl branched components, were detected in all samples. These components are included with the designation other components in Table 2.

## Hydroxy fatty acids

The LPS hydroxy fatty acid composition for samples from the methane-enriched HHD column A are presented in Table 4. A total of seven $\beta$-OH acids were detected with even-carbon numbered components predomi-nating. $\beta$-OH 14:0, $\beta$-OH 16:0, and $\beta$-OH 18:0 generally accounted for approxi-mately 80% of the total OH acid content. Differences were apparent in the relative levels of these three components.

## DISCUSSION

### Biomass

Bacterial biomass assessments for untreated surface soil (0-10 cm) and the top 10 cm from both the methane-enriched HHD soil column (A) and the azide-inhibited column (B) were close to values obtained at the closely related Lula, Oklahoma site by the acridine orange direct count method (AODC; 7.0-8.2 x $10^8$ cells/g wet weight; Balkwill, personal communication). These findings confirm the validity of the use of PLFA as a biomass assessment tool, as has been previously reported for estuarine and benthic marine sediments (38).

Relative to untreated surface soil, the microbial biomass was 2.3 times higher and 2.6 times lower for soil from the top 10 cm of the HHD columns and control azide-fed respectively. These cell number estimates are considerably higher than those reported for the AODC method for three shallow aquifers in Oklahoma (2.9-9.8 x $10^6$ cells/g dry weight; 2, 41). McCarty and co-workers (5, 23) and others (27, 42) have previously reported that biotransformation rates may be increased through the stimulation of indigenous bacteria by injection of a suitable primary substrate and required nutrients. The data presented here are in accord with this view and suggest that the degradation of short-chain halogenated hydrocarbons is accompanied by an increase in microbial biomass. Smith et al. (31) showed the bacterial biomass, estimated from extractable phospholipid, increased significantly in subsurface sediments in the recovered Vadose layer at sites polluted with creosote waste.

## Fatty acids

It is recognized that certain fatty acids are specific to bacteria and that different groups of bacteria can have different fatty acid compositions (14, 21). As a result, PLFA profiles have been used previously to determine microbial community structure (3, 12, 13, 15, 26, 36, 38). Differences observed for complex environmental samples have been subdivided through a bacterial chemotype approach, however, it is clearly of more use to obtain information on the proportions of metabolic groups within an environment. At the present time an increasing proportion of complex microbial consortia and environmental samples can be rationalized in terms of their fatty acid profiles into metabolic subgroups when detailed analysis, including determination of double-bond configuration and position, is performed. The fatty acid profiles obtained in this study will be discussed from this standpoint.

## Methanotrophs

The most significant feature, when fatty acid profiles of the methane-enriched HHD soil samples (column A) are compared to the control column (B) and control untreated surface soil, is the presence of the relatively novel monounsaturated components 18:1Δ10c, 16:1Δ8c and 16:1Δ10c. These fatty acids were absent in the non-HHD samples. Amounts of the former component increased with depth (Figure 1), constituting 28% of the PLFA at 8-10 cm in methane-enriched column A. The acid 18:1Δ10c has only been reported as a major component in methanotrophic bacteria, including Methylosinus trichosporium (22, 24). This component (18:1Δ10c) constituted ~50% of the total PLFA in M. trichosporium and 37% and 51% of the total PLFA in two related unclassified methanotrophs. Thus, from the data obtained for M. trichosporium, it can

be calculated that this bacterium or related bacteria account for between 32% and 56% of the total microbial biomass in the HHD soil column (A). As this novel signature fatty acid was below detection in both untreated control surface soil and soil from the control column (B), these data indicate that a significant change in the community structure has occurred in the HHD soil column relative to the other soils analyzed.

The second most abundant component detected in the methane-enriched column (A), 18:1Δ11c, was detected by Makula (22) as a minor component (11% to 18% of the total PLFA) in type II methanotrophs. In our analysis of the PLFA of four methanotrophs (24), 18:1Δ11c was the dominant component (84-89% of the total PLFA) in two strains of the type II methanotroph Methylobacterium organophilum. Thus, it appears that M. organophilum or related type II methanotrophs may also contribute a substantial proportion of this fatty acid and the overall microbial biomass in the HHD soil column (A). This acid (18:1Δ11c) is the most commonly detected bacterial $C_{18}$ monounsaturated fatty acid in many environments (12, 13, 15), and thus sources additional to type II methanotrophic bacteria may also be possible. At the present time, however, the significant increases in both the absolute and relative proportions of 18:1Δ11c, when the methane-enriched HHD soil column (A) is compared to the control column and untreated surface soil, are consistent with a large increase in the biomass of M. organophilum or related type II methanotrophs.

The $C_{18}$ monounsaturated fatty acids discussed in detail above are found in type II methanotrophs (24, 34), whereas $C_{16}$ components predominate in type I methanotrophs such as Methylomonas spp. The greater proportion of $C_{18}$ fatty acids in all samples from the upper 10 cm of the methane-

enriched HHD soil column (A) (Figure 2), thus, indicates that type II methanotrophs are more abundant than type I organisms.

Precise determination of double-bond location and geometry has enabled the clear distinction of signature fatty acids specific to methanotrophic bacteria to be performed. These data represent another example of the application of such chemical procedures to laboratory and environmental samples and the method can enable methanotrophic biomass to be monitored. Such methods, in many instances, provide information essential to the correct interpretation of increasingly complex data sets. In addition to the large community of methanotrophic bacteria, in the methane-enriched column (A), other microbial populations were also distinguished.

## Actinomycetes

Actinomycetes are commonly found in soils and have been reported to comprise 13% to 30% of the total microbial flora, depending on the season of the year (37). Members of Arthrobacter, Nocardioides, and other genera contain a number of 10-methyl fatty acids: 10 methyl 18:0 (tuberculosteric acid), 10 methyl 17:0, and 10 methyl 16:0 (25). Similarly, cyclopropane fatty acids were detected only in Actinomyces (19).

The presence of a series of 10 methyl branched fatty acids in all upper-10-cm soil samples analysed in this study (Table 3) is thus consistent with the presence of members of the Actinomycetes. The relative proportion of these components is significantly lower in the methane-enriched HHD soil column (A) than in either the column (B) or the untreated surface soil. A 20% contribution of the total PLFA in the HHD soil column is due to the 10 methyl branched fatty acid-containing Actinomycetes, according to calculations similar to those given above for monitoring methanotroph biomass and average Actinomyces fatty acid compositional data

(25). A higher contribution, 50% of the total PLFA, occurred in the control column (B) and untreated surface soil.

These calculations may represent an overestimation of this group of organisms, as it has recently been reported that Desulfobacter spp. also contain 10Me16:0 (33). Desulfobacter spp. typically contain low proportions of the longer chain homologues, 10Me17:0 and 10Me18:0. Thus, it seems reasonable to assume that a large proportion of the 10 methyl fatty acids are due to members of the Actinomycetes.

Other microbial groups

A number of fatty acids, reported in sulphate-reducing bacteria (4, 9, 33), were present in all samples (Table 3). The branched chain monoenoic fatty acids, i17:1Δ9c and i15:1Δ9c, are common to Desulfovibrio spp. (4, 9), and 10 methyl 16:0 is a major component in Desulfobacter as noted above. Iso 17:1Δ9c has also been detected recently in several Flexibacter (unpublished data). In the Flexibacter analyzed, i17:1Δ11 was also present in proportions similar to those of i17:1Δ9c. As i17:1Δ11 was not detected in this study the contribution from Flexibacter is probably minimal. If it is assumed that all the i17:1Δ9c detected can be attributed to Desulfovibrio spp., then this bacterial group contributes approximately 5%, 8%, and 10% of the total PLFA in the methane-enriched HHD soil column(A), the control column(B) and untreated surface soil, respectively.

It has been reported that certain atypical bacteria produce large amounts of branched-chain monoenoic acids (13). Other than for Desulfovibrio spp., i17:1Δ9c is not normally a dominant component. At the present time and based on the current literature, it has been assumed for the calculations above that Desulfovibrio spp. is the sole source of i17:1Δ9c.

Further studies are planned, including the analysis of PLFA from bacteria isolated from untreated surface soil, to clarify this point.

A minor contribution from eukaryotic organisms is occurring in all surface samples, as the $C_{20}$ PUFA 20:4Δ5 and 20:5Δ5 are specific to eukaryotes (3, 10, 36).

## LPS hydroxy fatty acids

Even carbon-numbered β-OH acids dominated the hydroxy acid profile for the methane-enriched HHD soil column (Table 4). Differences occurring in the upper 10 cm of the column can be rationalized as due to variations in the microbial, in particular methanotropic, community structure. The three major components, β-OH 14:0, β-OH 16:0, and β-OH 18:0, were the only components detected in four methanotrophs (24). These data add further supporting evidence to the view, based on the ester-linked PLFA profiles, that methanotrophic bacteria constitute a major proportion of the microbial biomass in the methane-enriched HHD soil column.

The PLFA and LPS hydroxy acid profiles reported have enabled the microbial biomass in a methane-enriched soil column and related control samples to be determined. Bacterial numbers, calculated using factors derived from bacterial monocultures (24, 39, Nichols et al., in press), were similar to those reported recently by the AODC method for closely related samples (2). These data thus validate the use of PLFA as a mechanism for determining microbial biomass in soil. More importantly, comparison of the PLFA profiles, particularly when precise determination of double-bond configuration is performed, has enabled bacterial community structure to be differentiated. The methane-enriched HHD column was found to be significantly higher in PLFA specific to type II methanotrophs. A

decrease in biomarkers for Actinomycetes and Desulfovibrio spp. was also
noted for the HHD soil relative to the other samples analysed.

Community structure information, as determined from PLFA, has provided
data on the effect of stimulating indigenous soil bacteria by addition of a
primary substrate and nutrients. The findings for this model system repre-
sent data that can be drawn upon as biotransformation processes similar to
the one described here are adapted to aquifers. Such a project is
presently underway in collaborative studies between our laboratories and
may help rationalize shifts in PLFA composition associated with pollution
in subsurface sediments.

## ACKNOWLEDGEMENTS

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Table 1  Phospholipid ester-linked fatty acid content and estimated cell
numbers for sediment samples from a methane enriched halogenated
hydrocarbon-degrading column and related samples.

| Sample | Sum of phospholipid ester-linked fatty acids nmoles/g (dry weight) | Number of cells/g[a] |
|---|---|---|
| Column A (methane-enriched)) | | |
| 0-10 cm[b] | 53.8 | $5.6 \times 10^9$ |
| 148-150 cm[c] | 0.61 | $6.3 \times 10^7$ |
| Column B (exposed to sodium azide) | | |
| 0-10 cm[c] | 8.93 | $9.3 \times 10^8$ |
| 148-150 cm[c] | 0.04 | $4.1 \times 10^6$ |
| Untreated surface soil[c] | 22.8 | $2.4 \times 10^9$ |

[a]: Determined using conversion factors described in text from White et al.
(14, 22), and Nichols et al. (24).

[b]: Mean of 10 samples.

[c]: Mean of 2 samples.

**Table 2**  Phospholipid normal ester-linked fatty acids from a methane-
enriched halogenated hydrocarbon-degrading sediment column and related
samples.

| Fatty acid | Percentage composition[a] | | | | | | | Column B: Control |
| | Column A: methane-enriched | | | | | | azide-inhibited |
| | 0-2[b] | 2-4 | 4-6 | 6-8 | 8-10 | 148-150 | 0-10 | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 12:0 | 0.22 | TR[c] | TR | TR | TR | 1.4 | -[h] | - |
| 13:0 | TR | TR | TR | TR | TR | 1.2 | - | - |
| 114:0 | 0.27 | 0.29 | 0.27 | 0.32 | 0.32 | | 0.20 | 0.20 |
| 14:0 | 1.3 | 1.5 | 1.6 | 1.4 | 1.3 | 1.4 | 1.1 | 0.37 |
| 115:1[d] | 0.20 | 0.37 | 0.20 | 0.19 | 0.2 | - | TR | TR |
| 115:0 | 2.5 | 2.9 | 2.7 | 2.8 | 3.0 | 2.6 | 5.4 | 5.9 |
| a15:0 | 1.2 | 1.5 | 1.4 | 1.4 | 1.7 | 2.1 | 2.3 | 2.6 |
| 15:0 | 0.73 | 0.45 | 0.44 | 0.46 | 0.42 | TR | 0.68 | 0.53 |
| 116:1Δ9c | 0.59 | 0.36 | 0.33 | 0.23 | 0.28 | - | 0.32 | 0.48 |
| 10Me15:0 | 0.20 | 0.19 | 0.15 | 0.16 | 0.14 | - | 0.31 | 0.29 |
| 116:0 | 2.1 | 1.5 | 2.1 | 1.5 | 1.6 | 3.7 | 3.8 | 3.8 |
| 16:1Δ7c | 0.50 | 1.1 | 0.53 | 0.44 | 1.0 | - | TR | 1.1 |
| 16:1Δ8c | NAQ[i] | NAQ | 2.4 | 3.3 | 2.4 | TR | - | - |
| 16:1Δ9c | 9.9 | 13.7 | 11.7 | 9.4 | 7.6 | 6.4 | 10.3 | 3.5 |
| 16:1Δ9t[e] | 1.4 | 1.3 | 1.5 | 1.3 | 1.1 | 2.6 | 1.9 | TR |
| 16:1Δ11c | 2.7 | 4.3 | 5.0 | 4.4 | 4.4 | 2.3 | 5.3 | 5.1 |
| 16:1Δ11t | TR | 0.35 | 0.13 | 0.10 | TR | - | - | - |

Table 2 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 16:0 | 9.2 | 8.8 | 8.6 | 8.4 | 8.0 | 62.1 | 13.7 | 11.2 |
| i17:1∆9c | 0.82 | 1.3 | 1.5 | 1.2 | 1.4 | 1.1 | 2.1 | 2.7 |
| 10Me16:0 | 1.6 | 2.0 | 2.0 | 2.0 | 2.3 | 2.3 | 5.4 | 5.7 |
| i17:0 | 0.92 | 1.0 | 1.1 | 1.1 | 1.2 | TR | 2.8 | 2.9 |
| a17:0 | 2.0 | 1.5 | 1.4 | 1.5 | 1.5 | 1.1 | 2.8 | 3.3 |
| cy17:0 | 1.5 | 1.9 | 2.3 | 2.2 | 2.0 | 1.3 | 4.8 | 2.3 |
| 17:0 | 0.62 | 0.41 | 0.36 | 0.26 | 0.36 | 1.1 | 0.81 | 0.66 |
| 10Me17:0 | 0.37 | 0.47 | 0.47 | 0.48 | 0.47 | – | 1.2 | 1.1 |
| 18:4∆6,9,12,15 | 2.5 | 1.9 | 1.5 | 1.9 | 2.3 | – | 0.38 | 0.24 |
| 18:2∆9,12 | 1.3 | 1.1 | 0.8 | 1.3 | 0.86 | TR | 0.90 | 4.8 |
| 18:1∆9c | 4.9 | NAQ | 3.2 | 0.63 | NAQ | – | 5.7 | 6.9 |
| 18:1∆10c | 16.1 | 21.5 | 20.3 | 25.6 | 27.9 | 1.6 | – | – |
| 18:1∆11c | 22.5 | 17.7 | 16.4 | 15.7 | 13.5 | 1.8 | 6.7 | 9.1 |
| 18:1∆11t | 0.22 | 0.23 | 0.46 | 0.32 | 0.30 | TR | 0.69 | 0.41 |
| 18:1∆13c | 0.61 | 1.2 | 0.94 | 0.93 | 1.1 | TR | 0.64 | 1.5 |
| 18:0 | 2.1 | 1.8 | 1.8 | 2.0 | 1.8 | 1.8 | 3.2 | 2.6 |
| 10Me18:0 | 1.4 | 0.82 | 0.92 | 0.99 | 1.0 | – | 2.2 | 1.8 |
| cy19:0 | 1.9 | 2.2 | 2.3 | 2.2 | 2.6 | 2.1 | 7.5 | 8.4 |
| 20:4∆5,8,11,14 | 1.4 | 1.5 | 1.5 | 1.3 | 1.3 | – | 0.20 | 1.2 |
| 20:5∆5,8,11,14,17 | 0.21 | 0.26 | 0.19 | 0.22 | 0.27 | – | TR | 0.36 |
| 20:3 | – | – | – | – | – | – | – | 0.72 |
| 20:1∆11c | – | – | – | – | – | – | 0.38 | 0.33 |
| 20:0 | TR | TR | TR | TR | TR | TR | 0.90 | 0.39 |
| others[f] | 4.0 | 2.6 | 2.4 | 2.3 | 4.4 | – | 5.4 | 7.6 |
| nmoles/g[g] | 42 | 61 | 50 | 74 | 42 | 0.6 | 8.9 | 23 |

Table 2 (con't)

[a]: Fatty acid composition is expressed in terms of the percentage of the total fatty acids. [b]: Depth in cm from top of column. [c]: TR, trace <0.1%. [d]: Position of unsaturation not determined because of insufficient sample material. Components present coelute with i 15:1Δ9c. [e]: 16:1Δ10c also present in several column A samples, but not quantified. [f]: Other components include trace amounts (<0.1%) of 14:1 (3 isomers), 15:1 (2 isomers) and 17:1 9c. [g]: Dry weight basis. [h]: – not detected. [i]: NAQ, present but not accurately quantitated.

Table 3 Monounsaturated fatty acids from a methane-enriched halogenated hydrocarbon-degrading sediment column (A). Gas chromatographic retention data and characteristic ion fragments of derivatized products formed by reaction of the fatty acids with dimethyldisulphide (DMDS).

| Fatty acid | $Rt^a$ | Ion fragments (m/z) of DMDS adducts | | |
| --- | --- | --- | --- | --- |
| | | M+ | $\omega$-fragment[b] | $\Delta$-fragment[c] |
| 115:1[d] | 17.79 | -[e] | - | - |
| 116:1$\Delta$9c | 19.74 | - | - | 217 |
| 16:1$\Delta$7c[d] | 20.34 | - | - | - |
| 16:1$\Delta$8c | 20.40 | - | 159 | 203 |
| 16:1$\Delta$9c | 20.44 | 362 | 145 | 217 |
| 16:1$\Delta$9t | 20.52 | 362 | 145 | 217 |
| 16:1$\Delta$10c | 20.52 | - | 131 | 231 |
| 16:1$\Delta$11c | 20.64 | 362 | 117 | 245 |
| 16:1$\Delta$11t | 20.77 | 362 | 117 | 245 |
| 117:1$\Delta$9c | 21.69 | - | 159 | 217 |
| 17:1$\Delta$9c | - | - | 159 | 217 |
| 18:1$\Delta$9c | 24.51 | - | 173 | 217 |
| 18:1$\Delta$10c | 24.57 | 390 | 159 | 231 |
| 18:1$\Delta$11c | 24.64 | 390 | 145 | 245 |
| 18:1$\Delta$11t | 24.76 | 390 | 145 | 245 |
| 18:1$\Delta$13c | 24.86 | 390 | 117 | 273 |

[a]: Retention time

[b]: ω-Fragment indicates fragment including aliphatic end of the molecule.

[c]: Δ-Fragment indicates fragment including carboxylic end of the molecule.

[d]: Identification based on GC retention data alone.

[e]: − Not detected in GC-MS analysis because of insufficient sample material.

Table 4    Lipopolysaccharide (LPS) β-hydroxy acids from a methane-enriched

halogenated hydrocarbon-degrading sediment column (A).

| β-hydroxy acid | Percentage composition[a] | | | | | |
|---|---|---|---|---|---|---|
| | 0-2[b]cm | 2-4cm | 4-6cm | 6-8cm | 8-10cm | 148-150cm |
| β-OH 10:0 | 6.5 | 4.6 | 10.5 | 4.7 | 5.5 | ND |
| β-OH 12:0 | 15.9 | 10.8 | 11.8 | 16.0 | 10.9 | TR |
| β-OH 13:0[c] | TR | TR | TR | TR | 5.6 | ND |
| β-OH 14:0 | 32.5 | 21.4 | 22.3 | 25.6 | 30.2 | TR |
| β-OH 15:0[c] | TR | TR | TR | TR | 4.7 | ND |
| β-OH 16:0 | 21.9 | 35.0 | 30.3 | 33.0 | 29.4 | TR |
| β-OH 18:0 | 23.1 | 33.1 | 25.1 | 20.7 | 13.5 | TR |
| nmoles/g[d] | 3.1 | 3.1 | 2.7 | 3.0 | 4.5 | 0.1 |

a:   β-OH acid composition is expressed in terms of the total β-hydroxy acids.

b:   Sediment depth (cm).

c:   Branched component.

d:   Dry weight basis.

FIGURE LEGENDS

Figure 1. Ratio of major fatty acids 18:1Δ10c and 18:1Δ11c versus sediment depth from a methane enriched halogenated hydrocarbon-degrading soil column (column A, without azide).

Figure 2. Relative proportions of (i) 16:0 and 16:1 isomers, closed symbols, and (ii) 18:0 and 18:1 isomers, open symbols. Circles, methane-enriched column A (halogenated hydrocarbon-degrading); squares, column B (exposed to sodium azide); triangles, untreated surface sediment.

PERCENTAGE of TOTAL FATTY ACIDS
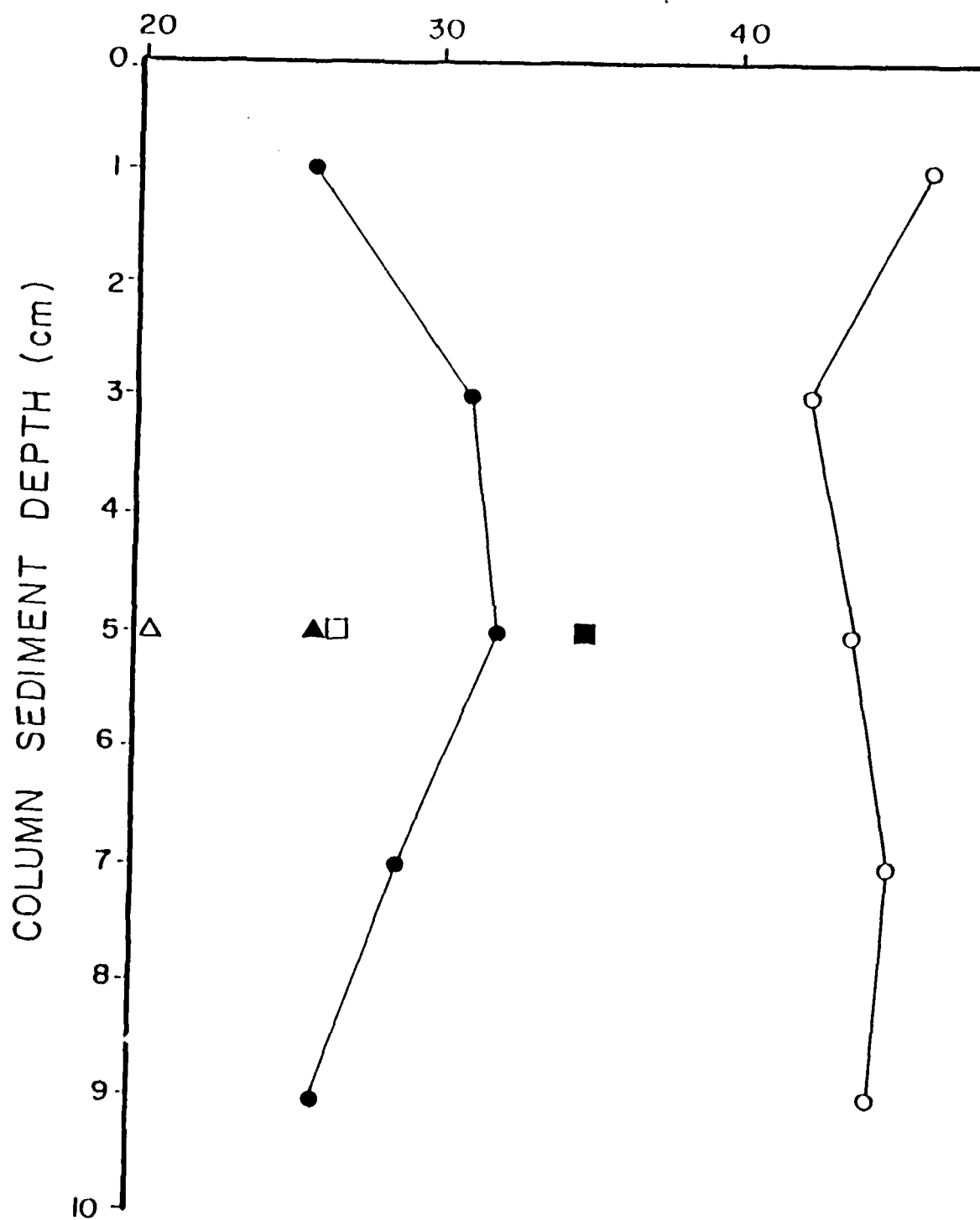
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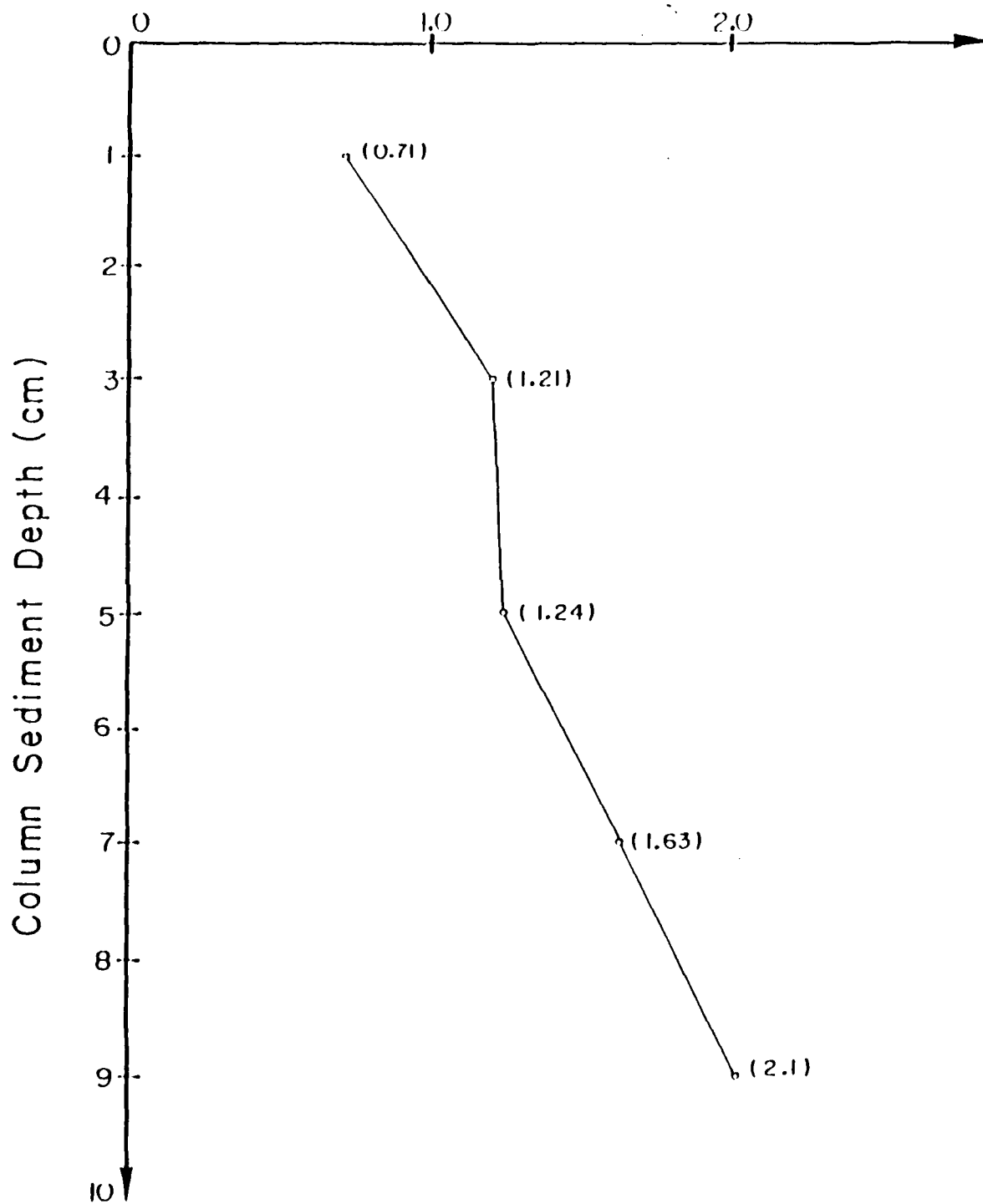
# Environmental Effects Testing with Quantitative Microbial Analysis: Chemical Signatures Correlated with *in situ* Biofilm Analysis by FT/IR

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## Abstract

Chemical measures for the biomass, community structure, nutritional status, and metabolic activities of the microbiota have shown a remarkable responsiveness to changes in the bulk fluid physical and chemical properties, the chemistry, biodegradability, and microtopology of the surfaces, as well as biological factors such as predation. Chemical analysis of the microbiota (bacteria, algae, fungi, protozoa, and micrometazoa with their extracellular products—0.5mm in diameter) does not require quantitative release of the organisms from surfaces or that the organisms are able to form colonies in subcultures. The sensitivity of the microbiota to changes in their habitat suggests that these chemical measures could provide a quantitative method for environmental effects testing. Changes in the marine benthic microbiota exposure to xenobiotics at the ug/l level or oil and gas well-drilling fluids demonstrate this sensitivity. These analyses do not destroy the vital interactions within microcolonies of mixed physiological types that characterize many environments. The chemical measures are destructive. Non-destructive analysis of biofilms of areas in the size range of microcolonies by Fourier transforming infrared spectrometry may ultimately provide the most effective method for environmental effects testing.

## HYPOTHESIS

The basis of this analysis assumes that the microbiota in a given environment faithfully reflect their optimal habitat. It further assumes that changes in the conditions of this habitat are rapidly and quantitatively revealed in the composition and activity of the microbiota. For convenience the term microbiota in this study includes the prokaryotes and the eukaryotes such as algae, protozoa, fungi, and micrometazoa that pass through a 0.5 mm mesh sieve.

## INTRODUCTION

The microbiota form the base of the food chain in soils and sediments. Recent studies indicate that the planktonic bacteria in the ocean may also represent a major feedstock to a large group of microflagellate protozoa that are in turn the food source of larger ciliate protozoa and zooplankton. There is no question that the microbiota represent the largest biomass of most environments. There are environments such as the hypersaline salt flats or hydrothermal springs or vents where microbes form the only biologically active components. These microbes are present in large numbers. For example the bacterial biomass of surface soils and sediments from environments as diverse as tropical estuaries or Antarctic marine sands is equivalent to greater than $10^9$ cells the size of *Pseudomonas fluorescens* per gram dry weight (White et al. 1984). Even such unlikely habitats as subsurface clay aquifer sediments recovered from depths as great as 420 meters below the surface may contain the equivalent of $10^6$ bacteria per gram dry weight (White et al. 1983).

This microbial biomass can persist in a dormant state with most of the individual organisms inactive for long periods or can spring to activity so rapidly that it becomes very difficult to estimate the native metabolic status. Fine-grained sediments often contain abundant reduced carbon energy sources and nutrients, but are limited in the concentration of terminal electron acceptors. The addition of oxygen that may occur when labeled precursors are added to the sediments to estimate metabolic activities can result in a significant "disturbance artifact". Such disturbance artifacts can be readily demonstrated by the incorporation of $H_3{}^{32}PO_4$ into phospholipids (Moriarty et al. 1985). Findlay et al. (1985) have developed a particularly sensitive measure of this disturbance by comparing the ratio of $^{14}C$-acetate incorporation into the prokaryotic endogenous storage product poly beta-hydroxy alkanoate (PHA) to phospholipid fatty acids (PLFA). In both these studies the onset of detectable increases in metabolic activity had occurred by the time the first sample could be taken.

There is a large and potentially active biomass of microbiota in nearly every environment in which life can survive. There is evidence that the community structure and metabolic activities of these microbes reflect the microhabitats of these organisms (White 1985). It follows that the introduction of chemical contamination

into microbial habitats should induce shifts in community structure and metabolic activities that could provide excellent quantitative microbial effects tests.

## The Problem

Microbes found in environments present a complex problem for assays. Even in the water column the classical methods of microbiology that involve the isolation and subsequent culturing of organisms on petri plates can lead to gross underestimations of the numbers of organisms detectable in direct counts of the same waters (Jannasch and Jones 1959). With sediments and biofilms the problems with classical methods are more severe. In addition to the problems of providing a universal growth medium in the petri plate, the organisms must be quantitatively removed from the surfaces and from each other. Direct microscopic methods that require quantitative release of the bacteria from the biofilm can have the problem of inconsistent removal from some surfaces. High speed blending of sediments to remove the microbiota prior to staining and direct counting in epifluorescent illumination was neither quantitative nor reproducible when compared to chemical assay of the muramic acid of the prokaryotic cell wall in one study of marine sediments (Moriarty 1980). Direct microscopy can be performed on the sediment particles or thin biofilms by making estimations for organisms rendered invisible by sediment granules or overlapping organisms in biofilms. The application of computer-based image enhancing can allow calculations of microbial biomass in complex assemblies (Caldwell and Germida 1984). This methodology works best when the density of organisms in the sediments or biofilms is low and overlapping is minimal. Even with computer enhanced image processing in direct microscopy, we are left with the problem that the *in situ* methods often fail because the morphology of a microbe offers little insight into the metabolic function or activity of the cells. Methane-forming bacteria for example come in all sizes and shapes (Zeikus 1977). The problem is further complicated by the fact that in many environments only a tiny fraction of the organisms is active at any one time and aside from the observation of bacterial doubling (Hagstrom et al. 1979), the morphology gives little evidence of the activity of the cells. Microscopic changes induced by showing the fraction of active cells by tetrazolium reduction and formazan

accumulation or nutrient stimulation in the presence of nalidixic acid with significant elongation of the cells appear to work in dilute planktonic environments (Maki and Remsen 1981). The most direct method of determining the proportion of active cells in a given biofilm involves a combination of autoradiography and electron or epifluorescence microscopy (Stanley and Staley 1977). All these methods require metabolic activity in the presence of the substrates and are subject to the limitations of density of organisms and thickness of the biofilm in the field of view. With the necessity for inducing metabolic activity there is a danger of inducing artificially high levels of activity with the addition of the substrates. Application of the extremely sensitive chemical methods has shown the induction of activity in the sedimentary microbiota when subjected to minimal disturbances (Findlay et al. 1985).

The attachment to and activity of microbes at surfaces is an extremely important feature of microbial ecology (Marshall 1976; 1984). Not only do microbes attach to surfaces, but many exist in consortia of multiple metabolic types. The best studied consortium is probably that of anaerobes that ferment complex plant polymers to volatile fatty acids, carbon dioxide, and methane in the rumen of vertebrates (Wolin 1979). Microcolonies of mixed bacterial types bound together with extracellular polymers are readily detectable in marine sediments by transmission electron microscopy (Moriarty and Hayward 1982). Isolation of the microbes in these consortia for viable counting or direct microscopic examination is often impossible and can provide little insight into the details of the interactions that take place. Since these consortia have much more versatile metabolic propensities than single species, it is important in environmental effects testing to preserve as much as possible the anatomy and metabolic interactions of these microcolonies. For that, a new type of analysis that does not involve quantitative removal of the microbes from surfaces or stimulation of new and possible artifactual metabolic activities is necessary.

## An Interim Solution

Our laboratory has been involved in the development of assays to define microbial consortia in which the bias of cultural selection of the classical plate count is eliminated. Since the total community is examined in these procedures without the necessity of removing

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the microbes from surfaces, the microstructure of multi-species
consortia is preserved. The method involves the measurement of
biochemical properties of the cells and their extracellular prod-
ucts. Those components generally distributed in cells are utilized
as measures of biomass. Components restricted to subsets of the
microbial communities can be utilized to define the community
structure. The concept of "signatures" for subsets of the com-
munity based on the limited distribution of specific components
has been validated by using antibiotics and cultural conditions
to manipulate the community structure. The resulting changes
agreed both morphologically and biochemically with the expected
results (White et al. 1980). Other validation experiments that
involved isolation and analysis of specific organisms and finding
them in appropriate mixtures, utilization of specific inhibitors and
noting the response, and changes in the local environment such as
the light intensity are summarized in a review (White 1983).

Phospholipids are found in the membranes of all cells. Under
the conditions expected in natural communities the bacteria con-
tain a relatively constant proportion of their biomass as phos-
pholipids (White et al. 1979c). Phospholipids are not found in
storage lipids and have a relatively rapid turnover in some sedi-
ments so the assay of these lipids gives a measure of the "viable"
cellular biomass (White et al. 1979b). The phosphate of the phos-
pholipids or the glycerol-phosphate and acid-labile glycerol from
phosphatidyl glycerol-like lipids that are indicators of bacterial
lipids can be assayed to increase the specificity and sensitivity of
the phospholipid assay (Gehron and White 1983).

The ester-linked fatty acids in the phospholipids are presently
both the most sensitive and the most useful chemical measures of
microbial biomass and community structure thus far developed
(Bobbie and White 1980; Guckert et al. 1985). The specification of
fatty acids that are ester-linked in the phospholipid fraction of the
total lipid extract greatly increases the selectivity of this assay as
most of the anthropogenic contaminants as well as the endogenous
storage lipids are found in the neutral or glycolipid fractions of the
lipids. By isolating the phospholipid fraction for fatty acid analysis
it proved possible to show bacteria in the sludge of crude oil tanks.
The specificity and sensitivity of this assay has been greatly in-
creased by the determination of the configuration and position
of double bounds in monoenoic fatty acids (Nichols et al. 1985;
Edlund et al. 1985) and by the formation of electron capturing

derivatives which after separation by capillary GLC can be detected after. chemical ionization mass spectrometry as negative ions at femtomolar sensitivities (Odham et al. 1985). This makes possible the detection of specific bacteria in the range of 10 to 100 organisms. Since many environments such as marine sediments often yield 150 ester-linked fatty acids derived from the phospholipids, a single assay provides a large amount of information. Combining a second derivatization of the fatty acid methyl esters to provide information on the configuration and localization of the double bonds in monounsaturated components provides even deeper insight. By utilizing fatty acid pattens of bacterial monocultures, Myron Sasser of the University of Delaware in collaboration with Hewlett Packard has been able to distinguish between over 800 strains of bacteria (Sasser 1985). Thus analysis of the fatty acids can provide insight into the community structure of microbial consortia as well as an estimate of the biomass.

Despite the fact that the analysis of PLFA cannot provide an exact description of each species or physiologic type of microbes in a given environment, the analysis provides a quantitative description of the microbiota in the particular environment sampled. With the techniques of statistical pattern recognition analysis it is possible to provide a quantitative estimate of the differences between samples with PLFA analysis. For example it proved possible to show that contamination of the subsurface aquifer sediment by creosote waste induced shifts in the microbial community structure in the vadose zone (Smith et al. 1985).

Potential problems with defining community structure by analysis of PLFA come with the shifts in fatty acid composition of some monocultures with changes in media composition or temperature (Lechevalier 1977) some of which were defined in this laboratory (Joyce et al. 1970; Frerman and White 1967; Ray et al. 1971). There is as yet little published evidence for such shifts in PLFA in nature where the growth conditions that allow survival in the highly competitive microbial consortia would be expected to severely restrict the survival of specific microbial strains to much narrower conditions of growth.

Analysis of other components of the phosholipid fraction give insight into the microbial community structure. "Signatures" (components restricted to subsets of the microbial community with similar physiological functions) for some of the microbial groups involved in anaerobic fermentations have been developed.

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The rate limiting step in fermentations is the degradation of poly-
mers. A second tier of microbes converts the carbohydrates and
amino acids released from the biopolymers into organic acids, alco-
hols, hydrogen, and carbon dioxide. These are the anaerobic fer-
menters and some of these organisms contain plasmalogen phos-
pholipids that are limited to this physiological class of anaerobes
in the microbial world (Goldfine and Hagen 1972). Plasmalogens
can be assayed by their resistance to alkaline methanolysis and
extreme sensitivity to mild acid (White et al. 1979d). Other groups
of anaerobic fermenters contain phosphosphingolipids with un-
usual sphingosine bases. These were detected in *Bacteroides* (Rizza
et al. 1970). Sphingosines are readily assayed in acid hydrolysates
of the polar lipids by their amino groups or by GLC of the long
chain bases (White et al. 1969).

Phytanyl glycerol diethers found in the Archaebacteria can be
assayed by high pressure liquid chromatography (HPLC) after
appropriate derivatization (Martz et al. 1983). C. Mancuso in this
laboratory has improved the sensitivity and resolution of the anal-
ysis of the diphytanylglycerol ether lipids of the methanogenic
bacteria by HPLC. She has also been able to show the presence of
isoprenologues of the aliphatic side chains of the diether lipids
using highly sensitive GC/MS techniques (Mancuso et al. 1985).

The sulfate-reducing bacteria contain lipids which can be
utilized to identify at least a portion of this class. Some contain a
unique profile of branched saturated and monounsaturated PLFA
(Edlund et al. 1985; Parkes and Taylor 1983; Taylor and Parkes
1983) that allows differentiation between those utilizing lactate
and those using acetate and higher fatty acids. Preliminary analy-
sis of sulfate-reducing bacteria by N. Dowling of this laboratory
strongly suggests that the majority of sulfate-reducing bacteria
found in marine sediments and in waters used in the secondary
recovery of oil are the acetate-utilizing strains. These organisms
are active even in fermentations in which there is no added sulfate
as they can recycle organic sulfur in the feed-stock (Smith and
Klug, 1981).

From the residue of the lipid-extracted biofilm, muramic acid,
a unique component of many bacterial cell walls can be recovered
(Findlay et al. 1983). Muramic acid in the bacterial cell wall exists
in a 1:1 molar ratio with glucosamine. Since the analysis gives
both glucosamine and muramic acid and the chitin walls of many
microeukaryotes yield glucosamine, the glucosamine to muramic

acid ratio gives insight into the prokaryote to eukaryote ratio. This complements the information developed from the ester-linked PLFA.

Gram-negative bacteria contain distinctive patterns of amide or ester-linked aliphatic and hydroxy fatty acids in the lipid A of their lipopolysaccharide wall polymers (Parker et al. 1982). This has proved to be an extremely valuable assay in the definition of gram-negative bacteria. With this assay it is possible to detect bacteria in mammalian tissue or secretions (Odham et al. 1985). Gram-positive bacteria often contain teichoic acid polymers as the substituted poly-glycerol or ribitol phosphate esters. Teichoic acid glycerol and ribitol can be released specifically by hydrolysis with cold concentrated hydrofluoric acid of the lipid extracted sediment (Gehron et al. 1984). With this assay it proved possible to show that contamination of subsurface aquifer sediments induces a shift from predominantly gram-positive to gram-negative based on the ratios of teichoic acid glycerol to phospholipid.

The methods described above provide insight into the biomass and community structure of microbial consortia at the time of the analysis. This is in some respects like the anatomy of a higher organism—it defines the potential of activities possible for this community. Phospholipids, adenosine nucleotides, muramic acid, and the lipopolysaccharide of dead bacteria are rapidly lost from marine sediments (Davis and White 1980; White et al. 1979b; 1979d; King et al. 1977; Moriarty 1977; Saddler and Wardlaw 1980). This indicates that the chemical markers provide good estimates for the standing viable or potentially viable microbiota.

## Nutritional Status

The nutritional status of biofilms or microbial consortia can be estimated by monitoring the proportions of specific endogenous storage compounds relative to the cellular biomass. The nutritional status of microeukaryotes (algae, fungi, or protozoa) in biofilms can be monitored by measuring the ratio of triglyceride glycerol to the cellular biomass (Gehron and White 1982). These microbes form triglyceride when exposed in a rich medium that is lost under conditions of starvation. With this assay it was possible to determine that amphipods existing in the estuary at the Florida State University marine laboratory have a triglyceride glycerol to phospholipid ratio typical of starvation. Toxicity testing with

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these amphipods should be done with organisms in the starving nutritional state if the exposure is to be in the estuary.

Certain bacteria form the endogenous lipid PHA under conditions when the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division (Nickels et al. 1979). A more sensitive assay based on GLC of the components of the PHA polymer demonstrated 4 and 5 carbon 3-OH acids in these polymers (Findlay and White 1983b). The sensitive assay of PHA has proved a useful means of defining the nutritional status of some heterotrophic microbes in various environmental habitats. The epiphytic microbiota on sea grass blades has a very high PHA/phospholipid ratio indicating that the leaf surface is an environment where the nutrients induce unbalanced growth (Herron et al. 1978). The chelating activity of the tannin-rich brown runoff water from the pine plantations of north Florida induces rapid accumulation of PHA in the estuarine detrital microbiota attached to oak leaves (Nickels et al. 1979). Recently it has been possible to study the rhizosphere of the rape plant *Brassica napus* (L.). Bacteria isolated from the roots when added to sterile soil and recovered show active formation of signature phospholipid fatty acids (PLFA) but no formation of PHA. These organisms recovered from rhizosphere show less growth and large amounts of PHA when compared to those attached to the roots (Tunlid et al. 1985b). Uncontaminated subsurface aquifer sediments show a microbiota with high levels of PHA relative to the phospholipids (White et al. 1983). Contamination of the subsurface sediments with aromatic phenols induces bacterial growth in the vadose zone with a decrease in the rate of PHA biosynthesis (Smith et al. 1985). The ratio of the rate of formation of PLFA to PHA from $^{14}$C-acetate has been shown to be an extraordinarily sensitive measure of the nutrient environment in the bacterial habitat (Findlay and White 1984; Findlay et al. 1985). With this measure it proved possible to detect the effects of raking intertidal sediments with a garden rake prior to measuring the rates of incorporation of $^{14}$C-acetate into PLFA and PHA if the isotope was carefully injected into cores of sediment. Other commonly utilized methods of measuring activity by forming slurries or filtering the isotopically labeled precursor through the core completely obscured the effects of the raking. Measuring the ratio of incorporation rates greatly increases sensitivity and allows measurement of the "disturbance artifact" involved in the application of labeled precursors to highly stratified

environments such as sediments (Findlay et al. 1985). In his Ph.D. studies R.H. Findlay showed a hierarchy of disturbance in a tidal sand bar. The undisturbed sediment showed the smallest values for the ratio of incorporation into PLFA/PHA. Increasing ratios based on the greater synthesis of PLFA (cellular growth) and lesser formation of PHA (carbon accumulation) for sands were found in the course of sand dollar feeding and bioturbation, and bioturbation in sting ray feeding pits. A still higher ratio was found in areas subjected to wind and tide disturbance. The highest ratio was found in the sediments slurried in the usual method of measuring microbial activities. Similar findings of increased rates of PLFA synthesis have been detected with disturbance of stratified sediments by measuring the incorporation of $H_3^{32}PO_4$ into phospholipids (Moriarty et al. 1985).

We have also developed assays for extracellular polysaccharide glycocalyx based on the specific content of uronic acids (Fazio et al. 1982). This assay has been utilized to show that poor growth conditions stimulate the formation of uronic acid containing exopolymers by a marine *Pseudomonas* (Uhlinger and White 1983). Uncontaminated subsurface aquifer sediments contain microbiota with very high levels of extracellular polysaccharides indicating poor nutrient conditions (White et al. 1983). The microfouling community formed on metal surfaces exposed to rapidly flowing seawater shows a rapid accumulation of uronic acid containing extracellular glycocalyx as a response to mechanical or chemical countermeasures (Nickels et al. 1981a; 1981c; White and Benson 1984). Preliminary evidence indicates that these polymers have a role in increasing marine sediment stability (Nowell et al. 1985).

## Metabolic Activity

The analyses described above all involve the isolation of components of microbial consortia. Since each of the components are isolated, the incorporation of labeled isotopes from precursors can be utilized to provide rates of synthesis of turnover in properly designed experiments. Measurements of the rates of synthesis of DNA with $^3H$-thymidine provide an estimate of the rates of heterotrophic bacterial growth if care is taken to utilize only short incubation times, if isotope dilution is utilized to estimate precursor concentration, and if DNA is purified prior to measurement (Moriarty and Pollard 1982). Incorporation of $^{35}S$-sulfate into

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sulfolipid can be utilized to measure activity in the microeuk-
aryotes (White et al. 1980; Moriarty et al. 1985). Incorporation of
$H_3^{32}PO_4$ into phospholipids can be utilized as a measure of the
activity of the total microbiota. The inhibition of phospholipid syn-
thesis in the presence of cycloheximide represents the microeu-
karyote portion of the lipid synthesis (White et al., 1980; Moriarty
et al. 1985). Measurement of rates of synthesis and turnover of
both carbon and phosphate in individual phospholipids showed
different turnover for the various lipids. Glycerol phosphoryl-
glycerol derived from phosphatidyl glycerol showed rates of loss
suggesting that the most rapid rates of growth of the detrital
microbiota were of the order of 100 hours (King et al. 1977).
Glycerol phosphorylcholine derived from phosphatidyl choline
showed extremely slow turnover in the detrital microbiota after
pulse chase exposure to $^{14}$C-labeled precursors. Grazing by am-
phipods markedly increased the loss of $^{14}$C-glycerol phosphoryl-
choline providing a quantitative estimate of grazing pressure
(Morrison and White 1980).

Analysis of signatures by GC/MS makes possible the utiliza-
tion of mass labeled precursors that are non-radioactive, have
specific activities approaching 100%, including isotopic marker for
nitrogen, and can be efficiently detected using the selective ion
mode in mass spectroscopy. The high specific activity makes possi-
ble the assay of critical reactions using substrate concentrations in
the biofilms that are just above the natural levels. This is not
possible with radioactive precursors. Improvements in analytical
techniques have increased the sensitivity of this analysis. Utiliz-
ing a chiral derivative and fused silica capillary GLC with chemi-
cal ionization and negative ion detection of selected ions, it proved
possible to detect 8pg (90 femtomoles) of D-alanine from the bacte-
rial cell wall (the equivalent of $10^5$ bacteria the size of E. coli
(Tunlid et al. 1985a). In this analysis it proved possible to repro-
ducibly detect a 1% enrichment of $^{15}$N-D-alanine in the $^{14}$N-D-
alanine.

## Detection of Effects on Microbial Consortia

The chemical methods for defining biomass, community structure,
nutritional status and metabolic activities described above can be
utilized to detect the effects of various disturbances on microbial
consortia in the environment.
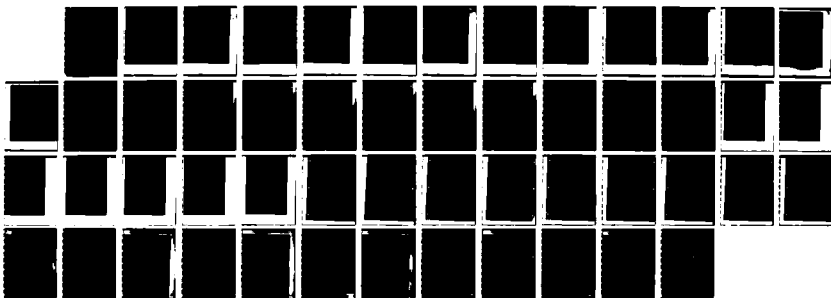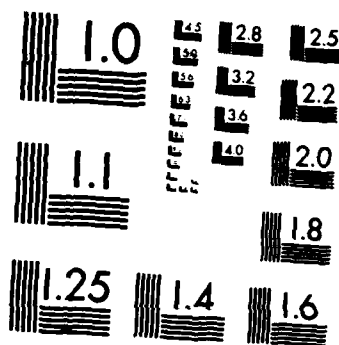
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

With these techniques it has been possible to show succession in marine biofouling films (Morrison et al. 1977; Nickels et al. 1981a), the effects of substratum biodegradability (Bobbie et al. 1978), the effects of substratum microtopology (Nickels et al. 1981b), the effects of mechanical disturbance (Nickels et al. 1981c), the effects of amphipod grazing and resource partitioning (Morrison and White 1980; Smith et al. 1982a), the effects of sand dollar bioturbation and predation (Findlay and White 1983a), the effects of essential elemental chelation (Nickels et al. 1979), and the effects of light (Bobbie et al. 1981) on microbial consortia.

In experiments utilizing an inoculum from marine sediments it was proved possible to manipulate the community structure of the benthic microbiota by shifting from aerobic to anaerobic conditions (Guckert et al. 1985). The fatty acid profiles of independent flasks showed reproducible shifts when manipulated identically and significant differences when manipulated with different treatments. The absence of long chain polyenoic fatty acids indicated the communities were predominantly prokaryotic and the differences in the phospholipid ester-linked fatty acids were primarily in the proportions of cyclopropane fatty acids and the proportions and geometry of the monounsaturated fatty acids.

In similar experiments a subsurface sediment inoculum was grown through two cycles of aerobic growth and compared to organisms from the same inoculum grown through two cycles of anaerobic growth with no supplement, or with sulfate or nitrate (D. Hedrick unpublished). Again there were reproducible shifts in the microbial community structure as reflected in the profiles of ester-linked phospholipid fatty acids. Hedrick also developed a sensitive assay for the respiratory quinones from subsurface samples utilizing HPLC with electrochemical detection. Benzoquinone isoprenologues are formed by microbes grown with high potential terminal electron acceptors such as oxygen or nitrate (Hollander et al. 1977). Naphthoquinones are formed by bacteria with respiratory systems with various potential terminal electron acceptors. As might have been predicted, the aerobic culture formed the most benzoquinone relative to naphthoquinone, the nitrate supplemented anaerobic culture formed less benzoquinone, the sulfate supplemented culture formed still less benzoquinone, and the anaerobic fermentation formed the least.

The biomass, community structure, and metabolic activity in the subsurface aquifer microbiota that resulted from contamina-

tion by improper disposal of creosote wastes all showed significant changes (Smith et al. 1985). Exposure to xenobiotics in the ug/l range markedly influenced the colonization of azoic marine sands in experiments designed to test the response to biocides in oil and gas well drilling muds (Smith et al. 1982b). These methods can be utilized with animals. The reef-building coral *Montastrea annularis* showed dose-response related shifts in amino acid pools, a drop in total phospholipid content, a shift from saturated to polyunsaturated fatty acids, a loss of triglycerides, and an increase in phospholipid fatty acids characteristic of bacterial infections on exposure to parts per million levels of oil and gas well-drilling muds (Parker et al. 1984).

It has been postulated for a long time that changes in rates of predation at the top of estuarine food chains would reverberate through the various trophic stages and finally affect the microbiota at its base. After developing methods for preserving sediment samples (Federle and White 1982) and sampling strategies for mud flats (Federle et al. 1983a) it was possible to show statistically significant differences in the community structure of the sedimentary microbiota by eliminating predation by the crabs and fish at the top of the food chain with properly designed caging experiments (Federle et al. 1983b). These experiments also showed significant differences in the benthic microbiota between continuous predation (crabs and fish caged inside) and the random predation of control areas. With the same type of technology it was possible to validate microcosms meant to mimic the estuarine waters nearby. The laboratory microcosms showed microbial biomass and community structures that were detectably different but the degree of difference was not large and did not increase with time when compared to the field in the system taken from a shallow, turbid, highly disturbed bay that is enriched by riverine runoff and is characterized by low macroscopic species diversity and high biomass. Microcosms prepared from a more stable, higher salinity, system with a much more diverse macroscopic community that is controlled by epibenthic predators showed a great difference from the field site. The differences between the microcosms in the laboratory and the field site increased drastically with time in this system (Federle et al. 1986).

The methods based on quantitative analysis of components of the microbiota and its extracellular polymers show responses to a variety of anthropogenic and natural perturbations clearly work

but they are not the solution. To truly understand the interactions of microbial consortia the analysis should be non-destructive, sensitive and continuous as well as have the resolution on the scale of micrometers—the sizes of microbial consortia themselves. Is such a technique available? Possibly . . .

## The Solution

The analysis of biofilms based on the isolation of chemical signatures is a destructive analysis and cannot be readily automated or utilized to give real-time monitoring of biofilms. The possibility of utilizing a non-destructive technique to monitor the chemistry of living biofilms is now possible with the Fourier transforming infrared spectrometer (FT/IR).

The infrared portion of the spectrum is extraordinarily rich in information regarding the vibrational and rotational motions of atoms in molecules. Not only can specific infrared absorption be assigned to particular types of covalent bonds but the modifications of these bonds by the local electronic environment can be detected in the details of the spectra (Bellamy 1958; Parker 1971). The infrared spectrum of a compound has long been accepted as one of the best nondestructive identification techniques.

One of the problems restricting the application of infrared spectroscopy has been that the atomic interactions sensed in the infrared portion of the spectrum are at relatively low energies and the detection is relatively inefficient. This has precluded the full usage of the power of the analysis using complex materials isolated from the environment.

The advent of fast computers has made possible a new type of infrared spectral analysis. This has provided the technology to utilize the far infrared portions of the spectrum, to follow rapid reaction rates with changes in spectral intensity, and to utilize different types of sample exposures such as photoacoustic spectroscopy. The secret lies in the array processor computers that can perform Fourier transformations so rapidly that interference spectroscopy can be possible.

The FT/IR has several advantages over conventional IR spectroscopy:

1. The Fellgett advantage results from the fact that the entire spectrum passes through the sample during the entire analytical interval. The spectrum is generated by the interference between

one portion of a split beam from a vibrating mirror. allowed to impinge on the interval. Conventional s scans the sample with a ments the signal from eac small portion of the analy ues to be generated throu the FT/IR both the signal cal interval. Under the c entire spectrum, during t ated, the random noise of quadrupling the number

2. In the throughput lizes the whole beam. C narrow slit of light on the cal beam. Thus only a s monochromator i utiliz throughput of the FT/IR n and thus a smaller portie

The combination of t the FT/IR gives an incr mid-IR spectral range of 1983).

3. The signal genera ally produced in the time frequency space in which Fourier transformation. tion of interference spect puters, it is actually an ad space are in general exp terms of sines and phase behaved, simple mathem space can be utilized to apodization etc. that woul ulations in frequency sp

4. For the interfero frequency domains the achieved using equal int sinusoidal interferogran part of the mirrors. The

one portion of a split beam that is "retarded" in that it is reflected from a vibrating mirror. In this way the entire interferogram is allowed to impinge on the sample throughout the entire analytical interval. Conventional spectrophotometers create a beam that scans the sample with a series of wavelengths. In these instruments the signal from each wavelength interval occurs for only a small portion of the analytical interval whereas the noise continues to be generated throughout the entire analytical interval. In the FT/IR both the signal and the noise occupy the entire analytical interval. Under the conditions of continuous analysis by the entire spectrum, during the time a signal of amplitude n is generated, the random noise of $n^{1/2}$ is also generated. This means that quadrupling the number of scans doubles the signal to noise ratio.

2. In the throughput or Jacquinot advantage the FT/IR utilizes the whole beam. Conventional spectrophotometers focus a narrow slit of light on the diffracting engine to create the analytical beam. Thus only a small portion of the light entering the monochromator is utilized in the analytical beam. The high throughput of the FT/IR means that a smaller beam can be utilized and thus a smaller portion of the sample can be analyzed.

The combination of the Fellgett and Jacquinot advantages in the FT/IR gives an increase in the signal to noise ratio in the mid-IR spectral range of 2.4 orders of magnitude (Griffiths 1975; 1983).

3. The signal generated by the FT/IR interferogram is actually produced in the time domain. To transform the signal into the frequency space in which spectra are usually perceived requires a Fourier transformation. Although this step prevented the utilization of interference spectroscopy until the advent of modern computers, it is actually an advantage. Since data described in Fourier space are in general expressed as successive approximations in terms of sines and phase (cosines) which are mathematically well behaved, simple mathematical manipulations on data in Fourier space can be utilized to correct for baseline shifts, overlapping, apodization etc. that would require complex mathematical manipulations in frequency space if they were even possible.

4. For the interferograms to be transformed from Fourier to frequency domains the signal is digitized. This digitization is achieved using equal intervals of optical path difference using the sinusoidal interferogram from a laser beam focused on another part of the mirrors. The interferogram is digitized once per wave-

length of the laser interferogram at the zero crossing which gives an extraordinary spectral resolution. Our instrument provides a spectral resolution that is continuously variable between 100 cm$^{-1}$ and 0.25 cm$^{-1}$. The best conventional IR spectrometers formerly gave resolutions of about 5 cm$^{-1}$. The extremely high resolution greatly increases the information content of the spectra.

5. Since a large capacity computer is required to transform the signals this computer can also be utilized to manipulate the signals. The generation of difference spectra by electronically subtracting before and after treatments allows small differences to be detected. These small differences can be further increased by plotting derivatives of the spectra.

6. The high capacity of the array processors allow for 64,000 Fourier transforms/second. This makes possible the online monitoring of successive scans. The computer can be programmed to discard scans that are outside specific limits set by the spectroscopist. This facilitates the use of repetitive scanning that greatly increases the sensitivity of analyses.

7. The high signal to noise ratio allows measurement in the far infrared region (1000 – 10 cm$^{-1}$). The weak vibrations that involve heavy metallic atoms can now be included in the spectra.

A summary of the use of FT/IR in microbial ecology has been published (Nichols et al. 1985b). The FT/IR examination by diffuse reflectance (DRIFT) of freeze-dried, powered bacterial monocultures shows two major groups. The first group is characterized by a dominant amide I (between 1690 and 1650 cm$^{-1}$) and amide II (1550 cm$^{-1}$) bands found in *Escherichia coli*, *Pseudomonas fluorescens*, *Desulfovibrio gigas*, *Staphylococcus aureus*, *Clostridium perfringens*, *Methylobacterium organophilium*, and *Methylosinus trichosporium* (both the latter grown on methane). Subtle variations in peak ratios of several groups could be utilized to differentiate between the different species. The second major group of organisms contained an enlarged carbonyl band at 1740 cm$^{-1}$. This group included *Bacillus subtilis*, *Methylobacterium organophilium* (grown on methanol), and *Nitrobacter winogradskyi*. These findings together with the powerful technique of subtraction of one spectrum from another suggest that DRIFT could be utilized to recognize differences in community structure. Preliminary experiments indicate that examination of planktonic microbiota on pre-extracted filters by DRIFT can be correlated with a detailed examination of the lipid content.

Two measures have been identified as markers for the micro-
bial nutritional status. The formation of PHA and the uronic
acid-containing exopolysaccharide glycocalyx are responses to nu-
tritional stress by bacteria. Both polymers can be detected with the
FT/IR. The polymers, such as gum arabic like the glycocalyx pro-
duced by *P. atlantica* show a prominent absorbance at 1150
cm⁻¹ for C–O stretch. The logarithm of the ratio absorbance at
C–O stretch to amide I gives an excellent correlation with mix-
tures of *E. coli* and gum arabic (Nichols et al. 1985b). This analysis
replaces a three week chemical tour-de-force involving GC/MS in
the analysis of bacterial glycocalyx. The DRIFT spectrum of *E. coli*
plus gum arabic and of *P. atlantica* induced to form polysaccharide
glycocalyx are similar in appearance. Accumulations of PHA in
bacteria or artificial mixtures of bacteria plus purified PHA show a
linear correlation with the ratio of the carbonyl stretch at 1750
cm⁻¹ to amide I. Using these recombination experiments as mod-
els, it proved possible to show DRIFT shifts in PHA and glycocalyx
in the biofilms formed in anaerobic fermenters that were supple-
mented with various amendments. For example amendments with
propionate or butyrate showed similar biofilms compared with
the unsupplemented or the biofilm of the digester amended with
nitrate.

The attenuated total reflectance cell (ATR) makes possible the
examination of living biofilms. The FT/IR can detect the vibration-
rotation interactions of biofilms about 300 um outside the surface
of the germanium crystal used in the ATR cell. With this system it
has proved possible to show that the carbohydrate rich initial
fouling polymer coats the germanium surface exposed to sterile
seawater in about 13 hours (J. Guckert of this laboratory). A
similar system has been utilized to follow the clotting sequence on
various plastics inserted into the blood stream of living sheep
(Gendreau and Jakobsen 1978). This is clearly the way to follow
biofilm formation and possibly to potentially monitor fermenta-
tions continuously. Not only is the FT/IR non-destructive, rapid
and sensitive but it is possible to decrease the beam size to diame-
ters approaching 10 um which is the scale of the microbial interac-
tions that must be monitored. Decreasing the area for analysis
requires that longer analysis times be utilized to achieve the same
sensitivity.

With the DRIFT analysis it proved possible to demonstrate
the reversible facilitation of corrosion of 304 stainless steel by the

non-sulfate reducing marine bacterium *Vibrio natriegens* and its extracellular material on the surface (Nivens et al. 1985). There was 15-fold increase in the corrosion current density measured electrochemically from the Tafel constants and polarization resistance that correlated with the colonization of the stainless steel disks by microcolonies of the bacteria. The colonization of the metal surface was detected both by direct microscopy after staining and epifluorescent illumination, scanning electron microscopy, and by an increase in the DRIFT absorbance at the amide I area centered at ~1660 cm$^{-1}$ corresponding to the bacterial protein. Maximum rates of corrosion were associated with the appearance of extracellular material with a spectral maximum centered at ~1440 cm$^{-1}$ similar to calcium hydroxide. Removing the biofilm, particularly the calcium hydroxide with its absorption at ~1440 cm$^{-1}$, decreased the corrosion current density 10-fold. In this instance both the presence of a non-sulfate reducing bacterium and its extracellular products reversibly facilitated corrosion of stainless steel in seawater.

Development of the FT/IR offers a potentially rapid and non-destructive method to examine the effects of toxicants on biofilms on the scale of the microbial consortia. The continued development of destructive analytical methods can be the essential validation for IR signatures.

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# Signature fatty acids in the polar lipids of acid-producing *Thiobacillus* spp.: methoxy, cyclopropyl, alpha-hydroxy-cyclopropyl and branched and normal monoenoic fatty acids

(Polar lipids; fatty acids; *Thiobacillus* ssp.; acid-producing bacteria; hydroxycyclopropyl fatty acids; methoxy fatty acids)

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## 1. SUMMARY

The polar lipids of 5 species of *Thiobacillus* were extracted and purified. An analysis of the fatty acid composition of the polar lipids documented the presence of methoxy, cyclopropyl, monounsaturated and hydroxycyclopropyl fatty acids of sufficiently unusual structure to serve as 'signatures' for the presence of these organisms in environmental samples. The structures of the unusual fatty acids of the polar lipids were confirmed by mass spectrometry (MS) after isolation by capillary gas chromatography (GC).

## 2. INTRODUCTION

The thiobacilli form a group of acid-producing, aerobic, Gram-negative bacilli with varying nutri-

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tional requirements. Reduced sulfur (elemental sulfur, hydrogen sulfide, or thiosulfate) is the characteristic substrate for oxidation by these organisms, which produce sulfuric acid as a metabolic end-product. These bacteria have the ability to survive external pH values between 1 and 4. Their acid production has been cited as a mechanism in the biodeterioration of concrete sewers [1] and utilized commercially in the recovery of metals from leach fields [2]. In both of these areas the quantitation of the role of thiobacilli in the environment, by analysis of a parameter which could be related to bacterial numbers, would be a useful measure and might replace time-consuming and often unreliable cultural enumeration techniques. Analyses of the membrane fatty acids indicated that unusual fatty acids and ubiquinones were present [3-7] and those findings, at least in principle, suggested that membrane fatty acids could be used as quantitative signatures for the thiobacilli.

In this study 'unusual' fatty acids in the polar lipids were detected. These polar lipid fatty acids (PLFA) are sufficiently unusual to be utilized as 'signature' patterns for the group as a whole. Preliminary examination of the PLFA of 5 species of acid-producing thiobacilli suggests that patterns of PLFA could be used to define the species. The PLFA pattern shifts with culture age. The proportions of 'unusual' PLFA such as the branched-cyclopropane, methoxy, and mid-chain branched acids accumulate with time.

## 3. MATERIALS AND METHODS

### 3.1. Materials

Solvents were distilled in glass and were of residue analysis grade or better (Baker Phillipsburg, NJ). Standards and derivatizing agents were purchased from Supelco (Bellefonte, PA), Applied Science (State College, PA), Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), and Pierce (Rockford, IL).

### 3.2. Organisms and cultural conditions

*Thiobacillus thiooxidans* ATCC19377 was grown in modified Starkey's medium [8] at 25°C on a rotary shaker. The medium contained $KH_2PO_4$, 3.0 g; $(NH_4)_2SO_4$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $Na_2S_2O_3 \cdot 7H_2O$, 10.0 g; with 1 ml trace elements [9] per l. The final pH was 3.5 after adjustment with sulfuric acid. Organisms were harvested in late stationary phase.

*T. thiooxidans* strain K-6 was isolated from the Hamburg sewer system [1] and was grown in the S-5 medium of Hutchinson et al. [10] supplemented with 10.0 g/l of $Na_2S_2O_3 \cdot 5H_2O$, at an initial pH of 4.5.

*Thiobacillus novellus* ATCC8093 was grown at pH 7.0 in Starkey's medium [8] with $Na_2S_2O_3$ as the energy source as described [11]. Organisms were grown on a rotary shaker at 25°C.

*Thiobacillus neapolitanus* strain B-2 was isolated and characterized [1], and grown in the medium labeled *Thiobacillus thioparus* of Vishniac and Santer [12] at an initial pH of 6.6.

*Thiobacillus intermedius* strain D-14 [1] was grown in the medium of Matin and Rittenberg [13] supplemented with $Na_2S_2O_3 \cdot 5H_2O$ 5.0 g, 0.1 g $CaCl_2$ per l with 0.1 mM biotin at an initial pH of 6.8.

*Thiobacillus acidophilus* ATCC27807 was grown in the medium of Guay and Silver [14], with glucose 10.0 g (added after autoclaving) and yeast extract 0.3 g/l at 25°C with stirring and an initial pH of 3.5.

Organisms were harvested by centrifugation at $10000 \times g$ for 15 min at 4°C, washed twice in phosphate buffer or growth medium minus energy source and lyophilized.

### 3.3. Extraction

The analytical sequence utilized for PLFA analysis is diagrammed in Fig. 1. The modified one-phase Bligh and Dyer extraction was utilized for all samples [15]. Duplicate samples were extracted separately and all data is expressed as the mean of two determinations. After the overnight separation of the lipid and aqueous phases in the second stage of the extraction, the organic fraction was filtered through fluted Whatman 2V filters and evaporated to dryness under a stream of nitrogen.

### 3.4. Polar lipid isolation

Silicic acid columns were prepared using 1 g Unisil (100-200 mesh; Clarkson Williamsport, PA) activated at 120°C for 60 min and pre-extracted with chloroform. Columns were prepared with the approximate ratios of 50:1 stationary phase to lipid (dry wt.) and 1.7:1 stationary phase column bed height:cross-sectional area. Total lipid was applied to the top of the columns in a minimal volume of chloroform. Sequential washes of 10 ml of chloroform, acetone, and methanol eluted the neutral, glyco-, and polar lipids, respectively. The polar lipid fraction was dried under a stream of nitrogen.

### 3.5. Mild alkaline methanolysis

The mild alkaline methanolysis procedure [15] was utilized to prepare methyl esters of the ester-linked fatty acids of the polar lipids.

### 3.6. Thin layer chromatography

Thin layer chromatography (TLC) on What-

Lyophilized Sample
↓
Lipid Extraction
↓
Silicic Acid
Fractionation
↘
Polar Lipids
↓
(Mild Alkaline Methanolysis)
↙ ↘
Lipids
↓
TLC
#1
↙ ↘
OH-FAME          Amino Lipids
FAME                    ↓
↙          (Acid Methanolysis)
TLC #2                 ↓
↙     ↘              ↙ ↘
OH-FAME    FAME        Lipid
↓         ↙   ↘         ↓
(TMSi)  (H₂) (DMDS)   (TMSi)
↓            (D-A)      ↓
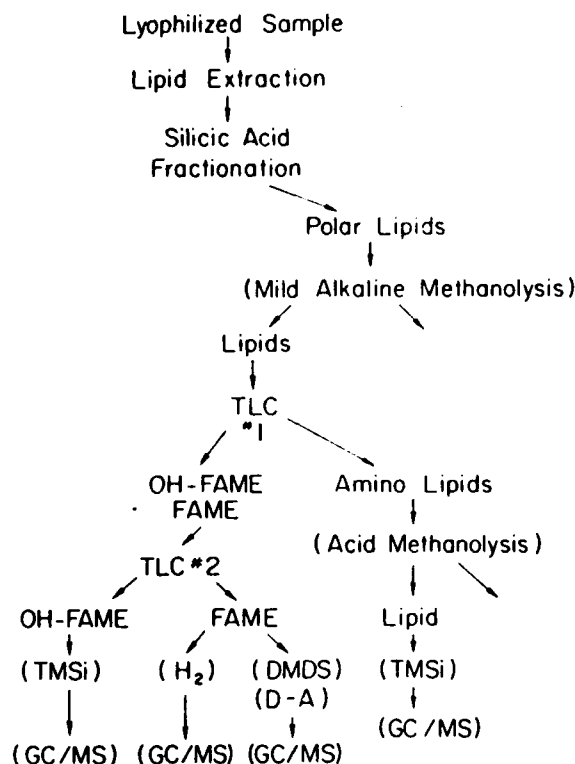↓             ↓      (GC/MS)
(GC/MS) (GC/MS) (GC/MS)

Fig. 1. Diagram of the isolation and analysis of the PLFA of the acid producing thiobacilli. TLC thin layer chromatography; OHFAME and FAME, hydroxy- and fatty acid methyl esters; TMSi, trimethylsilation; H₂, hydrogenation in acid; DMDS, formation of dimethyl disulfide adducts; D-A, formation of Diels-Alder adducts.

man K-6 silica gel (0.250 mm thick) was utilized for purification. The methyl esters from the polar lipid fraction were applied in a strip to the origin of the TLC plate that had previously been divided into a large mid-plate channel with two narrow channels on the edges. Authentic fatty acid methyl esters (FAME) and hydroxy fatty acid methyl esters (OHFAME) were applied to the outside channels and the plate placed in a tank for separation by ascending chromatography in a solvent of chloroform/methanol/water (55 : 35 : 6, v/v/v). The silica gel bands corresponding to the FAME and OHFAME ($R_1$ > 0.8) were scraped from the plate and the fatty acids recovered with chloroform/methanol (2 : 1, v/v). The position of the aminolipids was identified by spraying a por-

tion of the plate with 0.25% (w/v) ninhydrin in acetone/lutidine (9 : 1, v/v) [16]. The bands on the unsprayed portion of the plate were recovered in a Pasteur column and eluted with chloroform/methanol (1 : 1 and 2 : 1, v/v).

The FAME and OHFAME were separated using ascending chromatography with hexane : diethyl ether (1 : 1, v/v). The FAME band ($R_1$ 0.65) and OHFAME band ($R_1$ 0.25) were recovered and eluted with chloroform and chloroform/methanol (1 : 1, v/v).

### 3.7. Acid methanolysis

The aminolipids recovered from the TLC plates were subjected to acid methanolysis in anhydrous methanol/concentrated HCL/chloroform (10 : 1 : 1, v/v/v) after heating at 100°C for 1 h. The OHFAME were recovered in chloroform.

### 3.8. Derivatizations

Trimethylsilyl ethers of OHFAME were formed with N, O,-bis-(trimethylsilyl)-trifluoroacetamide (BSFTA) (Pierce) [17].

The position and geometry of the monounsaturation in the FAME and OHFAME was determined after two procedures. Dimethyl disulfide (DMDS) adducts were prepared as described [18]. These derivatives increase the resolution between cis and trans geometrical isomers in capillary GC. Diels-Alder adducts were prepared from the monounsaturated FAME and OHFAME utilizing 5,5-dimethoxy 1,2,3,4-tetrachlorocyclopentadiene (Sigma) using the modifications described for bacterial fatty acids [19]. In both these procedures the fragments from the omega (ω) or aliphatic end of the molecule and the delta (Δ) or carboxyl end of the molecule are clearly evident.

The position of the cyclopropane ring in the FAME can be determined after hydrogenation in the presence of Adam's catalyst of PtO₂ with the esters dissolved in methanol : glacial acetic acid (1 : 1, v/v) under a hydrogen atmosphere (140 kPa) at room temperature with mechanical agitation for 20-40 h in a Parr hydrogenation apparatus (Moline, IL) [20]. These derivatives give fragmentation patterns at branch points on either side of the original cyclopropane ring. Similar treatment of the 2-OHcy FAME did not yield

fragments allowing determination of the branch points.

## 3.9. Gas chromatography

Dry FAME or OHFAME were dissolved in hexane and the internal standard of methyl non-adecanoate added. Samples of 1.0 µl were injected onto a 50 m nonpolar, cross-linked methyl sili-cone-fused silica capillary column (0.2 mm i.d., Hewlett Packard) in a Varian 3700 GC. A 30-s splitless injection with the injection temperature at 250°C was used. Hydrogen at a linear velocity of 35 cm/s was the carrier gas with a temperature programme starting with an initial temperature of 80°C. The initial 20°C/min rise for 3 min followed by a 4°C/min rise for 30 min and an isothermal period for the remainder of the 40 min program was utilized. Detection was by hydrogen flame (FID) using a 30 ml/min nitrogen make up gas at a temperature of 290°C. An equal detector response was assumed for all components. Peak areas were quantified with a programmable laboratory data system (Hewlett Packard 3350 series) operated in an internal standard pro-gramme relative to known amounts of internal standard.

## 3.10. GC/MS

FAME and OHFAME were tentatively identi-fied by co-elution with authentic standards sup-plied by Supelco and Applied Science Labs. or previously identified laboratory standards. The analysis was performed on a Hewlett Packard 5996A GC/MS with a direct capillary inlet utiliz-ing the same chromatographic system except for the temperature programme which was begun at 100°C and increased to 280°C at 3°C/min for a total analysis time of 60 min. The electron multi-plier voltage was between 1400 and 1600 V, the transfer line maintained at 300°C, the source and analyzer maintained at 250°C, and the GC/MS was autotuned with DFTPP (decafluorotriphenyl-phosphine) at $m/z$ 502 with an ionization energy of 70 eV. The data was acquired and manipulated using the Hewlett Packard RTE 6/VM data sys-tem. Other conditions were as described previ-ously [20].

### 3.11. Fatty acid nomenclature

Fatty acids are designated as total number of carbon atoms: number of double bonds with the position of the double bond nearest to the aliphatic ($\omega$) end of the molecule indicated. This is fol-lowed by the suffix c for cis and t for trans configuration of monoenoic fatty acids. The pre-fixes i, a, or br indicate iso, anteiso, or branched (position undetermined). Mid-chain branching is indicated by the number of carbon atoms from the carboxyl ($\Delta$) end of the molecule and Me for the methyl group. Cyclopropane rings are indicated with the prefix cy and the position of the ring from the aliphatic ($\omega$) end of the molecule. Hy-droxy fatty acids are indicated by the number of carbon atoms from the carboxyl end of the mole-cule followed by the prefix OH. Methoxy fatty acids are given with the number of carbon atoms from the carboxyl end of the molecule followed by the prefix MeO.

## 4. RESULTS

### 4.1. Structural confirmation of the unusual fatty acids of Thiobacillus spp.

The data in Table 1 (1) show the mass spectral fragmentation patterns of monoenoic PLFA of 16, 17, 18 and 19 normal and 10 methyl branched 18-carbon monoenoic acids after fractionation by capillary GC. Fragmentation of the Diels-Alder adducts and dimethyldisulfide adducts showed the same position for the unsaturation in each acid. In part II of Table 1 the evidence for the position of the cyclopropane ring in 17- and 19-carbon chains, following ring opening hydrogenation, is pre-sented. Mass spectral evidence was obtained for a 21-carbon cyclopropane fatty acid which showed fragments at $m/z$ 55, 69, 74, 111, and 129 and also showed the expected GC retention time. Part III of Table 1 shows the evidence for α-hydroxy-cyclopropane fatty acids based on the fragmenta-tion of the trimethylsilated methyl esters. Parts IV and V of Table 1 show the evidence for methoxy 18- and 20-carbon fatty acids and mid-chain hy-droxy fatty acids.

### 4.2. PLFA patterns of Thiobacillus spp.

The proportions of the PLFA in 5 species and 2

Table 1

*Thiobacillus* spp. polar signature lipids

Characteristic ion fragments ($m/z$) of: I, Diels-Alder adducts and dimethyldisulfide adducts of monounsaturated fatty acids; II, products obtained following ring-opening hydrogenation of cyclopropyl fatty acids; III, hydroxy fatty acids; IV, methoxy fatty acids; V, mid-chain hydroxy fatty acids.

| Compound class/ fatty acid | Ion fragment ($m/z$) | | | | | |
|---|---|---|---|---|---|---|
| **I Mono-unsaturated fatty acid** [a] | Diels-Alder adducts | | | Dimethyldisulfide adducts | | |
| | [M-HCl] | Δ-Frag. [b] | ω-Frag. [c] | M [·] | Δ-Frag. [b] | ω-Frag. [c] |
| 16:1ω10 | 495 | 367 | 379 | [d] | | |
| 16:1ω7 | 495 | 409 | 337 | 362 | 217 | 145 |
| 16:1ω5 | - | | | 362 | 245 | 117 |
| 17:1ω9 | 509 | 395 | 365 | | | |
| 17:1ω8 | 509 | 409 | 351 | 376 | 217 | 159 |
| 17:1ω6 | 509 | 437 | 323 | 376 | 245 | 131 |
| i17:1ω5 [e] | - | | | | | 131 |
| 18:1ω9 | 523 | 409 | 365 | | | |
| 18:1ω7 | 523 | 437 | 337 | 390 | 245 | 145 |
| 18:1ω5 | 523 | 465 | 309 | 390 | 273 | 117 |
| 19:1ω9 | 537 | 423 | 365 | | 231 | 173 |
| 19:1ω8 | 537 | 437 | 351 | | 245 | 159 |
| 19:1ω7 | 537 | 451 | 337 | | 259 | 145 |
| 19:1ω6 | 537 | 465 | 323 | 404 | 273 | 131 |
| 10Me18:1ω6 | - | - | | 404 | 273 | 131 |
| 11Me18:1ω6 | - | - | | 404 | 273 | 131 |
| **II Cyclopropyl fatty acids** [f] | Products | | M [·] | | Other enhanced ions | |
| cy17:0(ω7,8) | 9Me16:0 | | 284 | | 157, 185 | |
| | 10Me16:0 | | 284 | | 171, 199 | |
| | 10Me18:0 | | 312 | | 171, 199 | |
| cy19:0(ω8,9) | 11Me18:0 | | 312 | | 185, 213 | |
| **III 2-OH-Hydroxy cyclopropane acid** [g] | [M-15] [·] | | [M-59] [·] | | Other major ion fragments | |
| 2-OHcy16:0 | 341 | | 297 | | 73, 89, 129, 159 | |
| 2-OHcy18:0 | 369 | | 325 | | 73, 89, 129, 159 | |
| 2-OHcy19:0 | 383 | | 339 | | 73, 89, 129, 159 | |
| **IV Methoxy fatty acids** [h] | Major ions | | | | Other ion fragments | |
| 10MeO18:0 | 157, 215 | | | | 55, 59, 69, 85 | |
| 11MeO18:0 | 143, 229 | | | | 55, 59, 69, 85 | |
| 12MeO20:0 | 157, 243 | | | | 55, 59, 69, 85 | |
| 13MeO20:0 | 143, 257 | | | | 55, 59, 69, 85 | |
| **V Mid-chain hydroxy fatty acids** [g,i] | Major ion | | | | Other ion fragments | |
| 11-OH19:0 | 215, 287 | | | | 73, 97, 129, 159 | |
| 13-OH19:0 | 187, 315 | | | | 73, 97, 129, 159 | |

[a] See MATERIALS AND METHODS for description of monounsaturated fatty acid derivatization procedures.
[b] Δ-Fragment indicates fragment including carboxylic end of the molecule.
[c] ω-Fragment indicates fragment including aliphatic end of the molecule.
[d] Not detected.
[e] Identified based on retention time, and the presence of a [Δ-32] fragment.
[f] Branched-chain cy21:0, brcy 20:0, and normal cy20:0 detected based on GLC retention time and mass spectral data before and after hydrogenation.
[g] Chromatographed as OTMSi ether.
[h] Prefix indicates position of methoxy group from the carboxylic end of the molecule. Major ions are due to fragmentation on either side of the methoxy group.
[i] Prefix indicates position of hydroxy group from the carboxylic end of the molecule. Major ions are due to fragmentation on either side of the OTMSi group.

strains of one species of acid-producing thiobacilli are listed in Tables 2 and 3. *T. neopolitanus* and strain K-6 of *T. thiooxidans* are clearly different from the others in having higher proportions of normal saturated PLFA such as 16:0, and 17:0 and low levels of cy19:0(ω8,9), which is a major

Table 2

Ester-linked fatty acid composition in the polar lipid of *Thiobacillus* spp., I. Non-hydroxy fatty acids

Fatty acids listed in order of elution from GLC. Trace components < 0.3%. A, B, and C are unidentified. * indicates unresolved components. MeO18:0 contains equivalent amounts of 11 and 10 methoxy branches. Total FAME represents the total amount of sample analyzed. Each value is the mean of 2 determinations.

| Fatty Acid | Strain (mol%) | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | *T. acidophilus* 27807 | *T. intermedius* D-4 | *T. neopolitanus* B-2 | *T. novellus* 8093 | *T. thiooxidans* 19377 | *T. thiooxidans* K-6 |
| 13:0 | | | | | 2.02 | |
| 14:0 | 0.10 | | 1.64 | | 0.28 | |
| 15:0 | | | 3.79 | | 0.22 | |
| 16:1ω10C | | | | | TRACE | |
| 16:1ω7C | 0.15 | 0.69 ⎱ | 15.1 * | TRACE | 1.05 | 9.19 |
| 16:1ω7T | | 0.18 ⎰ | | | 0.11 | |
| 16:1ω5C | | | 0.39 | TRACE | 0.22 | |
| A | | TRACE | TRACE | TRACE | TRACE | |
| 16:0 | 3.54 | 15.0 | 28.5 | 14.1 | 10.1 | 36.1 |
| i17:1ω5 | | | 0.17 | 1.24 | 0.21 | |
| 17:1ω9C | | 0.25 | | | TRACE | TRACE |
| 17:1ω8C | | | 1.55 | TRACE | 0.36 | TRACE |
| 17:1ω7C | | 0.26 | | | TRACE | TRACE |
| 17:1ω6C | 0.16 | TRACE | 0.59 | TRACE | 2.20 | TRACE |
| cy17:0(ω7,8) | | 9.46 | 1.20 | TRACE | 3.66 | TRACE |
| 17:0 | TRACE | 2.64 | 4.61 | 2.34 | TRACE | 5.56 |
| 18:1ω9C | TRACE | TRACE | TRACE | | TRACE | 0.54 |
| 18:1ω7C | 4.08 | 4.77 ⎱ | 37.0 * | 10.7 * ⎱ | 3.66 * ⎱ | 39.2 |
| 18:1ω7T | 0.25 | 1.86 ⎰ | | | | 0.31 |
| 18:1ω5C | 0.39 | 0.95 | 0.93 | 0.50 | 1.50 | TRACE |
| B | TRACE | 0.52 | | 0.31 | 0.99 | |
| 18:0 | 4.53 | 9.10 | 1.65 | 6.24 | 8.36 | 2.8 |
| MeO18:0 | 0.49 | 0.62 | | 0.29 | 0.69 | |
| 11Me18:1ω6 | 2.80 | 2.35 ⎱ | 0.4 * | 1.16 | 1.45 ⎱ | 0.7 * |
| 10Me18:1ω6 | 2.70 | 3.62 ⎰ | | 1.33 | 1.90 ⎰ | |
| 19:1ω9C | | | | | TRACE | TRACE |
| 19:1ω8C | TRACE | 1.04 | | 0.37 | TRACE | TRACE |
| 19:1ω7C | 0.62 | | | | TRACE | TRACE |
| 19:1ω6C | TRACE | | | | TRACE | TRACE |
| cy19:0(ω8,9) | 59.1 | 25.6 | 1.52 | 43.4 | 44.0 | 4.24 |
| brcy20:0 | 0.63 | TRACE | | | 1.51 | |
| cy20:0 | 12.2 | 11.6 | 0.34 | 9.14 | 8.11 | 1.35 |
| C | TRACE | TRACE | | TRACE | TRACE | |
| 13MeO20:0 | 1.43 | 1.45 | | 0.87 | 0.99 | |
| 12MeO20:0 | 1.42 | 1.41 | | 1.20 | 0.95 | |
| brcy21:0 | 3.00 | 3.09 | | 2.55 | 2.18 | |
| 21:0 | 1.25 | 2.60 | | 2.55 | 0.98 | |
| Total FAME (nmol) | 2500 | 435 | 769 | 48 | 313 | 1250 |

Table 3

Ester-linked fatty acid composition in the polar lipid of *Thiobacillus* spp. II. Hydroxy fatty acids

Fatty acids listed in order of elution from GLC. Trace components < 0.3%. A and B indicate 2-OH and 3-OHFAME, respectively.
* Indicates significant amounts of 3-OHFAME found in the aminolipid. The suffix A or B indicates the major isomer as determined
by GC/MS. Total OHFAME represents the total amount analyzed. Each value is the mean of 2 determinations.

| Fatty Acid | Strain (mol%) | | | | | |
|---|---|---|---|---|---|---|
| | *T. acidophilus* 27807 | *T. intermedius* D-14 | *T. neapolitanus* B-2 | *T. novellus* 8093 | *T. thiooxidans* 19377 | *T. thiooxidans* K-6 |
| A/BOH14:0 | 27.3 * B | 0.59 A | 5.95 B | 3.03 * | 12.1 * B | 5.74 * B |
| A/BOH15:0 | | | | | 1.57 * B | TRACE |
| AOHCY16:0 | | 6.44 | 28.2 | | | |
| A/BOH16:0 | 1.04 * B | 53.6 * A | 10.9 A | 9.22 * B | 31.9 * B | 25.0 * B |
| A/BOH17:0 | | 1.28 B | 3.23 A | 3.43 * A | 2.69 * A | 2.95 * A |
| AOHCY18:0 | 0.64 | 11.0 | 38.0 | TRACE | 1.37 | 1.61 |
| A/BOH18:0 | 66.2 * B | 5.27 B | 5.72 A | 65.9 * B | 5.73 | 22.6 * B |
| AOHCY19:0 | 0.80 | 15.3 | 8.02 | 5.40 | 43.4 | 42.2 |
| 11OH19:0 | 2.03 | 1.18 | | 2.37 | 0.43 | |
| 13OH19:0 | 2.03 | 0.91 | | 10.7 | 0.85 | |
| BOH22:0 | | | TRACE | | | |
| Ratio (×100) OHFAME/OHFAME + FAME | 6.25 | 12.3 | 1.50 | 13.6 | 28.5 | 15.2 |
| Total OHFAME (nmol) | 400 | 63 | 11.3 | 56 | 125 | 222 |

component of the other 2 species and strain 19377
of *T. thiooxidans* (Table 2). *T. neopolitanus* con-
tains the highest proportions of 2-OH cy16:0 and
2-OH cy18:0 in the PLFA (Table 3). *T. neopo-
litanus* contains the smallest proportion of
OHPLFA to PLFA of the 5 species. *T. thiooxidans*
K-6 is similar to *T. thiooxidans* 19377 in the high
content of 2-OH and 3-OH 16:0 and 2-OH cy19:0
than the other species. *T. intermedius* and both
strains of *T. thiooxidans* contain high proportions
of 3- and 2-OH 16:0 in contrast to *T. acidophilus*
and *T. novellus* which contain high proportions of
3- and 2-OH 18:0 (Table 3). The nonhydroxy
PLFA of *T. acidophilus, T. novellus, T. inter-
medius,* and *T. thiooxidans* 19377 show similarities
with relatively higher proportions of MeO18:0,
cy19:0(ω8,9), 10 and 11Me18:1ω6, 12 and
13MeO20:0, cy20:0, and brcy21:0 PLFA than
*T. neapolitanus* and *T. thiooxidans* K-6.

The tentative structures of each of the fatty
acids listed in Tables 2 and 3 have been confirmed
by GC/MS. The trimethylsilyl ethers (TMSi) of
the OHFAME showed an enhanced M-15 ion,

and fragments at $m/z$ 73, 89, and 159. The 2-
OTMSiFAME showed the diagnostic M-59 ion
[21], as well as major fragments at $m/z$ 103 and
129. The 3-OTMSiFAME show the definitive
fragment at $m/z$ 175 plus the rearrangement at
$m/z$ 133, and $m/z$ 159 and 131 [17,21]. Mid-chain
OTMSiFAME showed major ions attributable to
fragmentation either side of the OTMSi group.

*4.3. Shifts in the PLFA composition with time of
incubation*

The incubation of *T. acidophilus* in stationary
phase for 18 days resulted in a progressively de-
creased yield of cells and a marked shift in pro-
portions of the PLFA (Table 4). As the culture
ages the proportions of 'typical' PLFA such as
16:0 and 18:1ω7c decrease. No change in the
proportions of 14:0, 18:0 and the major compo-
nent cy19:0 is observed as the culture ages. The
proportions of the 'unusual' PLFA such as the
methoxy and mid-chain methyl-branched, the
branched cyclopropane, and cyclopropane 20-

74

| Fatty acid | 6 Days (mol%) | 12 Days (mol%) | 18 Days (mol%) |
|---|---|---|---|
| 14:0 | 0.11 | 0.21 | 0.10 |
| 16:1ω7C | 0.90 | 0.16 | 0.15 |
| 16:0 | 6.00 | 2.81 | 3.54 |
| 17:1ω6C | 0.23 | 0.10 | 0.16 |
| 18:1ω7C ⎫ | 18.0 | 12.2 | 4.08 |
| 18:1ω7T ⎭ | * | 0.34 | 0.25 |
| 18:1ω5C | 0.44 | 0.35 | 0.39 |
| 18:0 | 4.65 | 4.86 | 4.53 |
| MeO18:0 ⎫ | * ⎫ | * | 0.49 |
| 11Me18:1ω6 ⎬ | 1.08 * ⎬ | 6.78 * | 2.80 |
| 10Me18:1ω6 ⎭ | * ⎭ | * | 2.70 |
| cy19:0 | 68.6 | 51.8 | 59.1 |
| brcy20:0 | | 1.09 | 0.63 |
| cy20:0 | | 12.2 | 12.2 |
| 13MeO20:0 | | 1.15 | 1.43 |
| 12MeO20:0 | | 1.24 | 1.42 |
| brcy21:0 | | 2.62 | 3.00 |
| 21:0 | | 2.11 | 1.25 |
| Final pH | 2.55 | 2.75 | 2.65 |
| Yield (g/l) | 2.0 | 1.34 | 0.87 |

carbon acids as well as the 21-carbon acids increase as the incubation is prolonged.

## 5. DISCUSSION

### 5.1. Structural identification of unusual PLFA from Thiobacillus spp.

The unusual fatty acids from the phospholipids of the *Thiobacillus* spp. were tentatively identified from their retention times when compared to authentic standards. To define the structure of the PLFA, derivatives were made and the structures confirmed with their fragmentation patterns utilizing EIMS.

Previous work has emphasized the importance of the position and conformation of the double bond in monoenoic PLFA in defining specific groups of microorganisms. Gillan and Hogg [22] used the position of unsaturation in monounsaturated FAME to classify sedimentary micro-

organisms into 'chemotypes'. Nichols et al. [23,24] found unusual patterns of unsaturation in the monoenoic PLFA of the methane-oxidizing bacteria and the pathogen *Francisella tularensis*. These 'signature' patterns were sufficiently unique for detection of these organisms in environmental samples [23,25]. Guckert et al. [20] used changes in the proportions of monoenoic PLFA with defined positions of unsaturation to indicate the shifts in the benthic marine microbiota from aerobic to anaerobic growth. In a later study. Guckert et al. [26] showed the accumulation of *trans* monoenoic PLFA correlated with the starvation response in *Vibrio cholerae*.

Two methods of defining the position of unsaturation in PLFA have been utilized. The data in Table 1 show that both derivatizations confirmed the position of the unsaturation. The unusual unsaturation positions of ω 5, 6, 8, 10 together with the common ω 7 and 9 were all present in the PLFA of the acid-producing thiobacilli. Iso and mid-chain branched monounsaturated with ω 5 and 6 unsaturation were also detected (Table 1).

Cyclopropane fatty acids can be defined by creating the 2 mid-chain methyl branched isomers by hydrogenation of the cyclopropane ring in acid at elevated hydrogen pressure [20]. Cyclopropane rings are formed from monoenoic esters of specific phospholipids in the membranes of bacteria [27]. Their formation with the concomitant decrease in monoenoic PLFA occurs in monocultures that undergo metabolic stress such as stationary phase growth [28]. The same phenomenon has been detected in the benthic marine microbiota [26].

Methoxy PLFA are extremely unusual in microbiota. Great care must be taken to prevent their artifactual formation from cyclopropane fatty acid esters. Acid hydrolysis in methanol leads to the formation of methoxy derivatives of cyclopropane fatty acids [29]. The decreased yield of cyclopropane fatty acids in bacterial lipid preparations formed with acid hydrolysis has been known for a long time [30]. A methoxy fatty acid has been defined by GC/MS in the lipopolysaccharide-lipid A of *Thiobacillus ferrooxidans* [31]. It was this paper that encouraged us to examine the lipids of other *Thiobacillus* spp.. using the mild

alkaline methanolysis procedure, for signature PLFA that could be utilized in environmental studies.

Hydroxy fatty acids show retention time shifts after formation of TMSi ethers and give the distinctive fragmentation patterns with GC/EIMS described in Table 1. Both mid-chain and 2-OH and 3-OH acids were detected in the PLFA of the acid-producing thiobacilli.

### 5.2. Definition of unique PLFA

We believe this work provides the first documentation of the presence of 10- and 11-Me18:1ω6, cy19:0(8,9), 10- and 11-MeO18:0, 12- and 13-MeO20:0, 2-OHcy16:0, 2-OHcy18:0, and 11-OH and 13-OH19:0 in the polar lipids of bacteria. Knoche and Shively [6] reported 2-OHcy19:0(ω7,8) in the ornithine PLFA of *T. thiooxidans.*

### 5.3. Differentiation between species of Thiobacillus

Five species of acid-producing thiobacilli form patterns of PLFA that possibly could be utilized to differentiate between them (Tables 2 and 3). The two strains of *T. thiooxidans* show different patterns particularly in the nonhydroxy PLFA (Table 2). However both *T. thiooxidans* strains show high proportions of 2- and 3-OH 16:0 and 2-OHcy19:0 in the PLFA. The presence of high levels of a cy19:0 in the lipids of *T. thiooxidans* was noted by Levin [4]. Based on this study the presence of elevated levels of cy19:0(ω8,9) in the PLFA is so distinctive in some strains that it might be termed 'thiobacillic acid'. Levin [5] showed that the addition of traces of biotin greatly increased the proportions of the cy19:0 if *T. novellus* were grown autotrophically with thiosulfate or heterotrophically with lactate or glucose but not with citrate or yeast extract [5]. It should be noted, however, that both *T. neapolitanus* strain B-2 and *T. thiooxidans* strain K-6 utilized in this study contained only small amounts of this acid. Many more strains of these species and of other species of thiobacilli will need to be examined to establish that the PLFA patterns can be utilized to define the role of specific species in environmental samples.

The use of patterns of PLFA to define the community structure of microbial consortia have been utilized to show detrital succession, the effect of disturbance, or predation in marine sediments, the response to subsurface aquifer pollution and in environmental effects testing. The effects of shifts in the microbiota biofouling and corrosion studies have also been reviewed [32–35].

### 5.4. PLFA in the definition of microbial nutritional status

The data in Table 3 indicate that prolonged incubation of *T. acidophilus* results in a great increase in the 'unusual' long chain cyclopropane, methoxy-branched and mid-chain-branched monoenoic fatty acids. Preliminary experiments have shown that a number of these 'unusual' fatty acids can be readily detected in concrete samples exposed to these organisms [36] suggesting that they exist predominantly in the stationary phase in nature. Guckert et al. [20] were able to show higher relative proportions of cyclopropane as well as ω7 and *trans* monoenoic PLFA in the stationary phase of anaerobic cultures in manipulated microcosms. Branched-chain PLFA accumulated in the aerobic stationary phase [20].

### 5.5. Role of acid-producing thiobacilli in biodegradations

The correlation between the degree of biodeterioration of concrete and the activity of acid-producing *Thiobacillus* spp. has been shown using classical recovery and culture techniques [1,37]. Preliminary evidence from both corroding sewer systems and from a continuous culture apparatus designed to test the resistance of concrete samples to the corrosive activities of acid-producing bacteria shows that the degree of biodegradation appears to correlate with the presence of 'signature' PLFA of the acid-producing *Thiobacillus* spp., particularly *T. thiooxidans* [1,36,37]. The methodology described here will allow examination of an entire microbial ecosystem so the interactions between the acid-producing thiobacilli and the other organisms that potentiate their corrosive activities can be defined [38,39].

## ACKNOWLEDGEMENTS

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# Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts

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## Summary

Monounsaturated fatty acid double-bond position and geometry have been determined for microbial monocultures and complex microbial consortia by capillary GC-MS of their dimethyl disulphide (DMDS) adducts. The technique has permitted (*i*) chromatographic separation and positive identification of adducts derived from *cis/trans* isomers, (*ii*) characterization of long-chain monounsaturated components (up to 26:1), and (*iii*) the identification of a wide range of monounsaturated components derived from methanotrophic soil material. The methanotrophic soil sample contained a high relative proportion of the novel phospholipid ester-linked fatty acid 18:1 Δ10c. The DMDS procedure offers a simple and rapid approach that can be routinely applied to microbial fatty acids derived from environmental samples and monocultures.

## Introduction

Precise determination of monounsaturated fatty acid double-bond position and geometry is essential for the correct interpretation of complex data sets, in order that membrane fatty acids can be used as biomarkers in the fields of taxonomy, ecology,

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organic geochemistry and clinical microbiology. A number of derivatization procedures have been applied to sedimentary and microbially derived fatty acids to achieve structural identification [1-5]. These methods are not, however, applied routinely to environmental and microbial samples for a variety of reasons, such as lengthy sample workup, the method's failure to provide data for long-chain (greater than 24:1) components, and the degree of difficulty in interpreting relative proportions of *cis* and *trans* isomers.

A single-step derivatization procedure followed by gas chromatography-mass spectrometry (GC-MS) involving the simple and rapid iodine catalyzed addition of dimethyl disulphide (DMDS) to linear alkenes has recently been reported [6]. Similarly, the DMDS addition has also been used in the identification of standard monounsaturated acetates and fatty acid methyl esters derived from pheromone extracts of moth species [7]. The latter application achieved chromatographic separation of adducts derived from *cis* and *trans* isomers.

In this report we present data obtained by means of the DMDS procedure for monounsaturated fatty acids from microbial monocultures and complex soil consortia. Structural confirmation has been performed by capillary GC-MS of the adducts. The main criteria for the selection and use of this method were (*i*) confirmation of double-bond configuration for relatively novel monounsaturated fatty acids isolated from soil samples, (*ii*) distinction between *cis* and *trans* isomers, (*iii*) identification of long-chain monounsaturated, 24:1 and 26:1, fatty acids from a marine alga, (*iv*) the absence of contaminating byproducts, and (*v*) low cost and convenience of the reaction. The method has permitted the achievement of these goals.

## Materials and Methods

### Sample preparation

Sample extraction, fractionation of total lipid, and methylation of the phospholipid ester-linked fatty acids were as previously reported [8]. No further separation of the resulting methyl esters was required for this analysis.

### Gas chromatography

Fatty acid methyl esters (FAME) were taken up in hexane with methylnonadecanoate (19:0) as the internal injection standard. Separation of FAME was performed by high-resolution gas chromatography with a Hewlett Packard 5880A gas chromatograph (GC) equipped with a flame ionization detector. Samples were injected at 50°C in the splitless mode with a Hewlett Packard 7672 automatic sampler onto a non-polar cross-linked methyl silicone capillary column (50 m × 0.2 mm, i.d., Hewlett Packard). The oven temperature was programmed from 50 to 160°C at 10°C per min, then at 4°C per min to 300°C. Hydrogen was used as the carrier gas (1 ml/min). The injector and detector were maintained at 300°C.

Tentative peak identification, prior to GC-MS analysis, was based on comparison of retention times with those obtained for standards from Supelco Inc. (Bellefonte, PA) and Applied Science Laboratories Inc. (State College, PA) and previously

identified laboratory standards. Peak areas were quantified with a Hewlett Packard 3350 series programmable laboratory data system operated with an internal standard program.

## Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analyses were performed on a Hewlett-Packard 5996A system fitted with a direct capillary inlet. The same column type described above was used for analysis. Samples were injected in the splitless mode at 100 °C with a 0.5 min venting time, after which the oven temperature was programmed to 300 °C at either 3 or 4 °C per min. Helium was used as the carrier gas. MS operating parameters were: electron multiplier between 1300 and 1400 V, transfer line 300 °C, source and analyzer 250 °C, autotune file DFTPP normalized, optics tuned at m/z 502, electron impact energy = 70 eV. Mass spectral data were acquired and processed with a Hewlett Packard RTE-6/VM data system.

## Determination of fatty acid double-bond configuration

The DMDS adducts of monounsaturated FAME were formed according to methods similar to those described by Dunkelblum et al. [7] to locate the double-bond positions. A higher temperature was required to achieve complete reaction than reported for the monounsaturated acetates [7]. Samples in hexane (50 µl) were treated with 100 µl DMDS (gold label, Aldrich Chemical Co., Milwaukee, WI) and 1–2 drops of iodine solution (6% w:v in diethyl ether). The reaction took place in a standard 2 ml GC vial (Varian Assoc. Inc., Sunnyvale, CA) fitted with a teflon-lined screw-cap lid. After reaction at 50 °C in a GC oven for 48 h, the mixture was cooled and diluted with hexane (500 µl). Iodine was removed by shaking with 5% (w:v) aqueous sodium thiosulphate (500 µl). The organic layer was removed, and the aqueous layer reextracted with hexane:chloroform (4:1, v:v). The combined organic layers were concentrated under a stream of nitrogen prior to subsequent GC analysis. GC-MS analysis of the DMDS adducts showed major ions attributable to fragmentation between the two $CH_3S$ groups located at the original site of unsaturation (Fig. 1). Discrimination between cis and trans geometry in the original



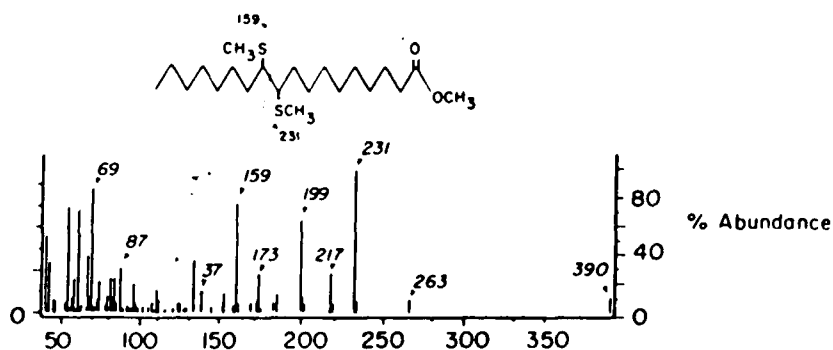Fig. 1. Mass spectrum of dimethyl disulphide adduct of 18:1Δ10c. Ions at m/z 390, 231 and 159 correspond to M+, Δ-fragment and ω-fragment of molecule. A minor contribution from 18:1Δ9c occurs with ions at m/z 217 and 173.

Scan Number

60    80    400    420    440    480

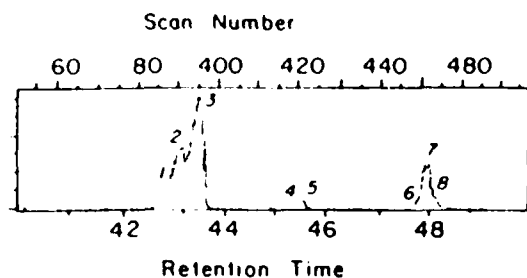

42    44    46    48

Retention Time

Fig. 2. Reconstructed ion chromatogram of dimethyl disulphide adducts from *Pseudomonas atlantica* monounsaturated fatty acids showing separation of *cis*- and *trans* derived adduct pairs (2, 3; 4, 5; 7, 8)

monounsaturated FAME was possible. The erythro isomer (originally the *trans* fatty acid) eluted after the threo isomer (originally the *cis* fatty acid) under the GC conditions employed (Fig. 2). The different positional isomers of the same geometry were chromatographically separated under the conditions used in this study.

*Fatty acid nomenclature*

Fatty acids are designated by total number of carbon atoms:number of double bonds, followed by the position of the double bond from the carboxylic ($\Delta$) end of the molecule. The suffixes c and t indicate *cis* and *trans* geometry. The prefix i refers to iso branching.

**Results and Discussion**

The DMDS adducts of monounsaturated FAME isolated from a methanotrophic soil column [9], *Pseudomonas atlantica* (a slime-producing bacterium) and *Nitzschia cylindrus* (a marine diatom) have been analyzed by capillary GC and GC-MS. Characteristic ion fragments for all monounsaturated FAME are shown in Table 1.

One major criterion for the selection of this technique was achievement of chromatographic separation of *cis* and *trans* acid adducts. A concurrent project in this laboratory has aimed to determine the physiological conditions under which bacteria produce *trans* fatty acids (Guckert and White, unpublished data). A simple and rapid technique that permitted positive identification of double-bond position and geometry and quantification of the two geometrical isomers was required. A reconstructed ion chromatogram showing the DMDS adducts of the monounsaturated FAME from *P. atlantica* is illustrated in Fig. 2. Separation of the adducts of the three *cis/trans* pairs, 16:1$\Delta$9c and t, 17:1$\Delta$9c and t, and 18:1$\Delta$11c and t, occurred under the chromatographic conditions employed for this GC-MS analysis. Better resolution for samples containing overlapping components (where one geometrical isomer dominated) was obtained by GC analysis using hydrogen as carrier gas. We have also noted a further increase in component resolution when a slightly more polar OV1701 column is used.

Application of the DMDS method to the long-chain monounsaturated

TABLE 1

CHARACTERISTIC ION FRAGMENTS OF DERIVATIVES FORMED BY REACTION OF THE PHOSPHOLIPID MONOUNSATURATED FATTY ACID METHYL ESTERS OF MICROBIAL MONOCULTURES AND CONSORTIA WITH DIMETHYL DISULPHIDE (DMDS)

| Fatty acid methyl ester | Rt[a] | Peak number[b] | Ion fragments (m/z) of DMDS adducts | | | |
|---|---|---|---|---|---|---|
| | | | M | ω-fragment[c] | Δ-fragment[d] | Source[f] |
| i15:1Δ9c | 17.79 | | | | 217 | M |
| i16:1Δ9c | 19.74 | | | | 217 | M |
| 16:1Δ7c | 20.34 | 1 | | 173 | | P, M |
| 16:1Δ8c | 20.40 | | | 159 | 203 | M |
| 16:1Δ9c | 20.44 | 2 | 362 | 145 | 217 | P, N, M |
| 16:1Δ9t | 20.52 | 3 | 362 | 145 | 217 | P, N, M |
| 16:1Δ10t | 20.52 | | | 131 | 231 | M |
| 16:1Δ11c | 20.64 | | 362 | 117 | 245 | M |
| 16:1Δ11t | 20.77 | | 362 | 117 | 245 | M |
| i17:1Δ9c | 21.69 | | | 159 | 217 | M |
| 17:1Δ9c | 22.43 | 4 | 376 | 159 | 217 | P, M |
| 17:1Δ9t | 22.55 | 5 | 376 | 159 | 217 | P |
| 18:1Δ9c | 24.51 | 6 | | 173 | 217 | P, M |
| 18:1Δ10c | 24.57 | | 390 | 159 | 231 | M |
| 18:1Δ11c | 24.64 | 7 | 390 | 145 | 245 | P, M |
| 18:1Δ11t | 24.76 | 8 | 390 | 145 | 245 | P, M |
| 18:1Δ13c | 24.86 | | 390 | 117 | 273 | M |
| 24:1Δ13c | 36.07 | | - | 201 | 273 | N |
| 24:1Δ15c | 36.16 | | - | 173 | 301 | N |
| 26:1Δ15c | 39.69 | | - | 201 | 301 | N |
| 26:1Δ17c | 39.79 | | - | 173 | 329 | N |

[a] Retention time.

[b] Peak numbers refer to Fig. 2.

[c] ω-Fragment indicates fragment including aliphatic end of the molecule.

[d] Δ-Fragment indicates fragment including carboxylic end of the molecule.

[e] -, Not detected in GC-MS analysis because of insufficient sample material.

[f] Source of lipid: M, methanotrophic soil column; P, *Pseudomonas atlantica*; N, *Nitzschia cylindrus*.

phospholipid FAME of *N. cylindrus* confirmed the presence of 24:1Δ13, 24:1Δ15c, 26:1Δ15c and 26:1Δ17c. The DMDS procedure has previously been applied to the acetates of monounsaturated fatty acids ranging from 12 to 18 carbon atoms long. The data presented here extend the reported working range of this method to monounsaturated fatty acids containing 26 carbon atoms. The use of shorter capillary columns than those utilized in this study will extend this range further. The procedure reported here will permit the identification of long-chain monounsaturated fatty acids from the pathogenic bacterium *Fransicella tularensis*. This bacterium has been previously reported to contain long-chain monounsaturated acids up to 26:1 [10, 11]. Similarly, we plan to apply the procedure routinely to the identification of long-chain components isolated from Antarctic benthic organisms and sediments. Our preliminary studies have indicated the presence of several of these relatively novel long-chain monounsaturated components (unpublished data).

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As a further test of the DMDS and subsequent GC-MS procedure, analysis of FAME obtained from the phospholipid fraction of a soil column exposed to natural gas (95% hydrocarbons, 77% methane [9]) was undertaken. Table 1 also includes the sixteen monounsaturated FAME that were positively identified in the soil material. The confirmation of the rarely reported fatty acid 18:1Δ10c by mass spectrometry (Fig. 1), as the major monounsaturated component in the phospholipid fraction, is consistent with the enrichment of a methanotrophic population. This component, to our knowledge, as yet has only been reported as a major phospholipid ester-linked fatty acid in *Methylosinus trichosporium* [12, 13]. Further interpretation of data obtained on fatty acids, including other monounsaturates, present in the soil sample could permit a more complete understanding of the microbial community structure.

Use of the DMDS derivatization procedure followed by GC-MS analysis offers a simple and rapid method for the determination of monounsaturated fatty acid double-bond position and geometry. The method has permitted chromatographic separation and positive identification of *cis/trans* isomers, long-chain components up to 26:1, and several relatively novel components from a complex environmental sample. These separations and identifications are prerequisites to the full exploitation of fatty acid profiles. Although polyunsaturated FAME are similarly derivatized by this procedure, the chromatographic conditions used for this study did not permit elution of these DMDS adducts. The data presented indicate that the DMDS procedure can be easily and routinely applied to monounsaturated fatty acids derived from microbial monocultures and complex consortia.

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# Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for methane-oxidizing bacteria

(Methylotrophs; fatty acid structural verification; signature lipids)

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## 1. SUMMARY

The extractable ester-linked and the lipopoly-saccharide (LPS) normal and hydroxy fatty acids of the methylotrophic bacteria *Methylosinus trichosporium* OB3B, *Methylobacterium organophilum* XX, grown on methane and methanol, *Mb. organophilum* RG and *Methylomonas* sp. were analysed by capillary gas chromatography-mass spectrometry (GC-MS). Precise monounsaturated double bond position and geometry was determined by GC-MS analysis of the derivatized fatty acids. The three species were readily distinguished based on the extractable fatty acid and LPS hydroxy acid profiles. Type I and Type II methylotrophs can be separated based on the presence of 16-carbon and 18-carbon monoenoic fatty acids in the two groups of organisms, respectively. Relatively novel components, 18:1ω8c, 18:1ω8t, 18:1ω7t and 18:1ω6c were present in *Ms. trichosporium*, and 16:1ω8c, 16:1ω8t, 16:1ω7t, 16:1ω5c and 16:1ω5t were detected in *Methylomonas* sp. These specific lipids may be used, together with other components, as signatures for these methylotrophic bacteria in manipulated laboratory and environmental samples.

## 2. INTRODUCTION

The interactions of aerobic and anaerobic bacteria in aquatic sediments are extremely complex [1]. Methane-oxidizing bacteria occupy an extremely important niche in biogeochemical cycling [2]. Recent studies [3] have shown that a large proportion of the complex carbon polymers produced during photosynthesis is anaerobically degraded in sediments by consortia of microorganisms to yield methane. The methane produced is mineralized to carbon dioxide at microaerophilic sites by a group of methane-oxidizing organisms that may be important in nitrogen fixation [4]. The estimated 1-10% of the methane that escapes into the atmosphere is extremely important in the greenhouse effect [5]. Recognition of these facts suggests that methods to identify and quantify the presence of methane-oxidizing bacteria would be extremely useful.

Biochemical methods that quantitatively recover and measure cellular components have been successfully applied to a wide range of environmental samples. Microbial biomass and community structure can be determined by measuring properties common to all cells and identifying

specific signature or biological marker lipids respectively [6-9]. The advantages of chemical procedures, when compared with classical enumeration procedures, have been described [6].

Specific signature lipid components have been reported for a number of metabolic groups of anaerobic bacteria. The methane-producing bacteria contain di- and tetra-phytanyl glycerol ether phospholipids [10]. The potential for the analysis of ether lipids has been demonstrated [11]. Similarly biological marker branched chain mono-unsaturated and midchain methyl branched fatty acids have been found in sulphate-reducing bacteria [12,13].

The methane oxidizers or methylotrophic bacteria utilize methane and have been detected in a variety of natural environments [14]. In the few reports available on the lipid composition of methylotrophs, a number of unusual lipid components has been noted in the extensive membrane systems in these bacteria [15]. Methane oxidizers have been classified into two groups [16] on the basis of the type of intracytoplasmic membranes they possess and of the metabolic pathway for the utilization of $C_1$ compounds. 4-Methyl and 4,4-dimethyl sterols containing an unusual ring double bond at the $\Delta^{8(14)}$ position were isolated from *Methylococcus capsulatus* [17]. Makula [18], in the first report of double bond positional isomers in both group I and II methane-utilizing bacteria, detected *cis* and *trans* $\Delta^8$, $\Delta^9$, $\Delta^{10}$ and $\Delta^{11}$ monounsaturated fatty acids in 4 organisms.

The overall aim of this study was to provide specific lipid components which may be used to monitor for methane-oxidizing bacteria in manipulated laboratory and field samples. The normal and nonhydroxy fatty acid composition of three methylotrophs is reported here, to extend the range of signature lipids for this group of organisms.

# 3. MATERIALS AND METHODS

## 3.1. Bacteria and culture conditions

*Methylomonas* sp. strain 761 was isolated from a pond in a rice field in South China [19]. This bacterium is an unusual type I methanotroph that

assimilates formaldehyde via the hexulose monophosphate pathway but, unlike other type I methylotrophs, it possesses a complete tricarboxylic acid cycle [20]. Cells were grown with methane as the sole carbon and energy source [20].

*Mb. organophilum* strains XX and RG, are facultative type II methanotrophs, and were grown in AMS basal salts medium [21] with an atmosphere of methane and air (1:4, v:v) or with methanol (0.1%, w:v) in the absence of methane.

*Ms. trichosporium* OB3B [22], was grown in the AMS medium under the same conditions as *Mb. organophilum* XX.

All cultures were grown at 30°C and harvested by centrifugation at 10 000 × g for 15 min (4°C) in an RC-5 superspeed refrigerated centrifuge (Dupont Instruments Co., Newton, CT) and washed twice in potassium phosphate buffer, pH 7.0, by centrifugation.

## 3.2. Lipid extraction and fractionation

Lipids were extracted with $CHCl_3$–MeOH (2:1; v/v) from lyophilized cells (50–100 mg) in glass test tubes fitted with teflon-lined screw caps. The extraction was repeated and the combined extracts were partitioned with distilled water. The lower $CHCl_3$ layer, containing the total lipid material, was collected. Total lipid was separated into 3 general classes: neutral, glyco- and phospholipid, by silicic acid column chromatography [23,24]. The fractions were collected in test tubes, dried under a stream of nitrogen, and stored at −20°C until further analysis. The mild alkaline methanolysis procedure [25] was applied to the phospholipid-containing methanol fraction. Lipopolysaccharide (LPS) normal and hydroxy fatty acids were obtained from the solvent-extracted cellular residue using the method described by Moss [26].

## 3.3. Gas chromatography

Fatty acid methyl ester samples were taken up in hexane with methylnonadecanoate (19:0) as the internal standard. Initial identification of individual normal and hydroxy (as the corresponding TMSi ethers) fatty acid components was performed by high resolution gas chromatography using a Hewlett Packard 5880A gas chromatograph equipped with a flame ionization detector.

Samples were injected at 50°C in the splitless mode on a non-polar, crosslinked methyl silicone capillary column (50 m × 0.2 mm i.d., Hewlett Packard). The oven was temperature programmed from 50°C to 160°C at 5°C per min, then at 2°C per min to 300°C. Hydrogen was used as the carrier gas.

Tentative peak identification, prior to GC–MS analysis, was based on comparison of retention times with data for standards obtained from Supelco Inc. (Bellefonte, PA) and Applied Science Laboratories Inc. (State College, PA) and previously identified laboratory standards. Peak areas were quantified using a Hewlett Packard 3350 series programmable laboratory data system operated in an internal standard program. Replicate analyses were performed for *Ms. trichosporium* and *Mb. organophilum* XX (grown on methane and methanol). Fatty acid compositional data reported for these 3 samples is the mean of the 2 analyses. Standard deviations for individual fatty acids were in the range 0–30%, typically < 5%.

### 3.4. Gas chromatography–mass spectrometry

GC–MS analyses were performed on a Hewlett Packard 5995A system fitted with a direct capillary inlet. The same column type as the one described above was used for analyses. Samples were injected in the splitless mode at 100°C and the oven was programmed from 100°C to 300°C at 4°C per min. Hydrogen was used as the carrier gas. MS operating parameters (peak finder and SIM modes) were: electron multiplier 1400 or 1600 V, transfer line 300°C, source and analyser 250°C, autotune file DFTPP normalized, optics tuned at m/z 502, MS peak detect threshold = 300 triggered on total ion abundance, electron impact energy = 70 eV.

### 3.5. Determination of fatty acid double bond configuration

Monounsaturated fatty acid methyl esters were converted to their corresponding diols by reaction with osmium tetroxide followed by the previously reported sample work-up [24,27]. GC–MS analysis of the bis-trimethylsilyl ether derivatives of the dihydroxy fatty acids showed major ions attributable to fragmentation between the derivatized hydroxy groups (Table 1). Discrimination between

Table 1

Monounsaturated fatty acids of methane oxidizing microbes and the characteristic ion fragments of derivatized dihydroxylation products formed by reaction of the fatty acids with osmium tetroxide

| Parent acid [a] | Ion fragments (m/z) of dihydroxy trimethylsilyl ethers | |
|---|---|---|
| | ω-fragments [b] | Δ-fragments [c] |
| 16:1ω9 | 215 | 231 |
| 16:1ω8 | 201 | 245 |
| 16:1ω7 | 187 | 259 |
| 16:1ω5 | 159 | 287 |
| 17:1ω8 | 201 | 259 |
| 18:1ω9 | 215 | 259 |
| 18:1ω8 | 201 | 273 |
| 18:1ω7 | 187 | 287 |
| 18:1ω6 | 173 | 301 |

[a] Includes *cis* and *trans* geometrical isomers
[b] ω-Fragments indicates fragment including aliphatic end of the molecule.
[c] Δ-Fragments indicates fragment including carboxyl end of the molecule

*cis* and *trans* geometry of the double bond in the original monoenoic fatty acid methyl ester is possible using the ditrimethylsilyl derivatives. The erythro isomer (originally the *cis* fatty acid) elutes after the threo isomer (originally the *trans* fatty acid). The different positional isomers of the same geometry were chromatographically separated under the GC–MS conditions used in this study.

### 3.6. Fatty acid nomenclature

Fatty acids are designated as total number of carbon atoms: number of double bonds followed by the position of the double bond from the ω(aliphatic) end of the molecule. The prefixes i and a refer to iso and anteiso branching respectively and the prefix OH indicates a hydroxy group at the position indicated. The suffixes c and t indicate *cis* and *trans*. The Δ nomenclature system for fatty acids indicates the position of the double bond from the carboxyl end of the molecule.

## 4. RESULTS

### 4.1. Phospholipid and LPS fatty acid concentrations

The concentration of phospholipid ester-linked

normal and LPS normal and hydroxy fatty acids in *Methylosinus trichosporium* and *Methylobacterium organophilum* XX grown on both methane and methanol (Tables 1, 2 and 3) were essentially equivalent. The phospholipid normal fatty acids were in the range 45–60 $\mu$mol/g (dry weight basis) for the two bacteria. This is typical of many eubacteria [28]. The concentrations of LPS normal and hydroxy fatty acids in these two organisms were determined to be between 0.35–0.55% and 0.12–0.77%, respectively, of the phospholipid ester-linked normal fatty acids. Phospholipid hydroxy fatty acids were not detected in any of the bacteria analysed.

### 4.2. Neutral lipid components

A number of unidentified cyclic components was detected, by GC and GC–MS analysis, in the neutral lipid-containing chloroform fraction. Further work is planned to determine the structure of these components and to assess their potential to act as signatures for these methylotrophs.

### 4.3. Extractable normal fatty acids

The phospholipid ester-linked fatty acid profiles of the 5 cultures of methane oxidizers fall in the 14–19-carbon range (Table 2) typically found in bacteria [29,30]. The 3 species analysed each showed distinctive fatty acid profiles, with several

Table 2

Extractable normal fatty acids of methane-oxidizing bacteria

*M. trichosporium* and *M. organophilum*, grown on methane, also contained trace amounts of i and a15:0 and 17:0. *Methylomonas* sp. (unfractionated lipids analysed) also contained TR amounts of 14:1 (2 isomers) and 15:0 (0.8%).

| Fatty acid | Percentage composition [a] | | | | |
|---|---|---|---|---|---|
| | Type I | Type II | | | |
| | *Methylomonas* sp. 761 | *Methylosinus trichosporium* OB3B | *Methylobacterium organophilum* XX | | *Methylobacterium organophilum* RG |
| | (Methane [b]) | (Methane) | (Methane) | (Methanol) | (Methane) |
| 14:0 | 17.9 | TR | TR | – | – |
| 16:1ω9c | TR | – | – | – | – |
| 16:1ω8c | 30.0 | – | – | – | – |
| 16:1ω8t | 1.5 | – | – | – | – |
| 16:1ω7c | 17.8 | 9.0 | TR | TR | – |
| 16:1ω7t | 5.6 | 1.1 | – | – | – |
| 16:1ω5c | 16.3 | – | – | – | – |
| 16:1ω5t | 0.2 | – | – | – | – |
| 16:0 | 7.0 | 1.6 | 1.6 | 2.2 | 2.4 |
| 17:1ω8c | – | TR | – | – | – |
| 17:0 | – | – | 0.6 | 0.3 | 1.0 |
| 18:1ω9c | 0.3 | – | 0.4 | TR | 0.6 |
| 18:1ω8c | – | 49.2 | – | – | – |
| 18:1ω8t | – | 9.3 | – | – | – |
| 18:1ω7c | 1.5 | 25.1 | 89.3 | 86.7 | 83.7 |
| 18:1ω7t | – | 2.0 | 3.3 | 3.4 | 1.8 |
| 18:1ω6c | – | 1.9 | – | – | – |
| 18:0 | 0.5 | 0.8 | 5.0 | 7.4 | 10.4 |
| cis/trans | 9.0 | 6.9 | 27.7 | 25.5 | 46.8 |
| Total $\mu$mol/g [c] | ND | 45 | 60 | 66 | ND |

TR trace < 0.1%; ND, not determined.

[a] Fatty acid composition is expressed in terms of the percentage of total acids.

[b] Carbon source.

[c] Dry weight basis.

novel components detected in *Ms. trichosporium* and *Methylomonas* sp. (Table 2).

*Ms. trichosporium* contained higher relative levels (approx. 90% of the total fatty acids) of 18 carbon monounsaturated components (Table 2). The acids in decreasing order of abundance were: 18 : 1ω8c, 18 : 1ω7c, 18 : 1ω8t, 18 : 1ω7t and 18 : 1ω6c. Positive identification of these components, in particular the rarely reported 8 and 6 isomers, was only possible after derivatization of the parent acids and subsequent analysis of the products by GC–MS.

*Methylomonas* sp. 761, the only group I organism analysed in this study, was readily distinguished from the group II bacteria (Table 2). This bacterium contained a high relative abundance of 16 carbon monounsaturated components (71% of the total fatty acids), in contrast to the major proportions of 18 carbon monounsaturates present in the group II bacteria *Mb. organophilum*

(both strains) and *Ms. trichosporium* The mono-unsaturates found in *Methylomonas* sp. in decreasing order of abundance were: 16 : 1ω8c, 16 : 1ω7c, 16 : 1ω5c, 16 : 1ω7t, 16 : 1ω8t, 16 : 1ω5t and 16 : 1ω9c. The saturated acid 14 : 0, not present in either group II organism, was also a major component (18% of the total acids) in *Methylomonas* sp.

The phospholipid fatty acid profiles obtained for *Mb. organophilum* XX grown on either methane or methanol and *Mb. organophilum* RG were generally very similar (Table 2). The 18 carbon monounsaturate 18 : 1ω7c, which is characteristic of the anaerobic desaturase biosynthetic pathway, was the dominant acid in all analyses (85–90% of the total fatty acids). The next most abundant components were 18 : 0, 18 : 1ω7t and 16 : 0. The fatty acid profile obtained for *Mb. organophilum* was simpler than that obtained for the other 2 organisms, with only the 4 acids listed above present at greater than 1% of the fatty acids.

Table 3

Lipopolysaccharide normal fatty acid composition of methane-oxidizing bacteria

| Fatty acid | Percentage composition [a] | | | | |
| --- | --- | --- | --- | --- | --- |
| | Type I | Type II | | | |
| | *Methylomonas* sp. 761 | *Methylosinus trichosporium* OB3B | *Methylobacterium organophilum* XX | | *Methylobacterium organophilum* RG |
| | (Methane [b]) | (Methane) | (Methane) | (Methanol) | Methane) |
| 14:0 | 6.2 | 7.1 | 5.0 | 1.1 | – |
| i15:0 | – | 3.9 | 4.5 | – | – |
| a15:0 | – | 11.0 | 2.8 | – | – |
| 15:0 | 2.2 | 0.3 | 0.8 | – | – |
| i16:0 | – | 4.7 | 0.4 | 0.3 | – |
| 16:1ω7c | – | 7.0 | 0.9 | 1.9 | – |
| 16:1ω7t | – | TR | TR | – | – |
| 16:0 | 56.7 | 8.7 | 4.3 | 1.0 | 4.7 |
| 18:1ω8c | – | 36.0 | – | – | – |
| 18:1ω8t | – | 15.6 | – | – | – |
| 18:1ω7c | – | 8.2 | 69.0 | 79.0 | 79.6 |
| 18:1ω7t | – | – | 3.8 | 4.5 | 6.9 |
| 18:0 | 32.7 | 2.7 | 8.8 | 9.9 | 8.8 |
| Total nmol/g [c] | ND | 40 | 55 | 36 | ND |

[a] Fatty acid composition is expressed in terms of the percentage of the total normal acids
[b] Carbon source.
[c] Dry weight basis.
ND, not determined; TR, trace < 0.1%

Table 4

Lipopolysaccharide hydroxy fatty acid composition of methane-oxidizing bacteria

| Fatty acid | Percentage composition [a] | | | | |
|---|---|---|---|---|---|
| | Type I | | Type II | | |
| | Methylomonas sp 761 | Methylosinus trichosporium OB3B | Methylobacterium organophilum XX | | Methylobacterium organophilum RG |
| | (Methane) [b] | (Methane) | (Methane) | (Methanol) | (Methane) |
| β-OH 14:0 | TR | | 59.1 | 56.1 | 53.8 |
| β-OH 16:0 | 99.9 | 14.9 | 1.4 | 1.2 | 1.7 |
| β-OH 18:0 | | 85.1 | 39.5 | 42.7 | 44.4 |
| Total nmol/g [c] | ND | 77 | 19 | 12 | ND |

[a] Hydroxy fatty acid composition expressed in terms of the percentage of total hydroxy acids

[b] Carbon source

[c] Dry weight basis

TR, trace. < 0.1%; ND, not determined

## 4.4. LPS normal fatty acids

The LPS normal fatty acid profiles obtained for *M. organophilum* and *Ms. trichosporium* were similar to the phospholipid profiles, although slight differences were apparent (Table 3). In contrast, *Methylomonas* sp., which contained substantial amounts of 16 carbon monounsaturates in its extractable fatty acids, showed only saturated straight-chain LPS fatty acids.

## 4.5. LPS hydroxy fatty acids

LPS hydroxy fatty acids were detected in all 3 species (Table 4). These findings, to our knowledge, represent only the second report of β-hydroxy acids in methane-oxidizing bacteria and further extends the range of Gram-negative organisms in which β-OH acids are found. In a previous study [35], β-hydroxy acids were not detected in *Ms. trichosporium* or *Mb. organophilum* [35]. The latter organism contained β-OH 14:0. The constituent hydroxy acids of *Methylomonas* sp. have not been previously reported. The three β-OH acids detected in this study were: β-OH 14:0, β-OH 16:0 and β-OH 18:0. The 3 genera analysed were readily separated based on the relative levels of the 3 β-OH acids (Table 4).

## 5. DISCUSSION

The extractable normal fatty acids and LPS normal and hydroxy fatty acids have been analysed in detail using capillary GC and GC-MS. Members of the genera *Methylosinus*, *Methylobacterium* and *Methylomonas* have been distinguished based on the profiles obtained. The finding of a number of novel fatty acids, 18:1ω8c, 18:1ω8t, 18:1ω7t and 18:1ω6c in *Ms. trichosporium* is in accord with previous work by Makula [18]. He detected both *cis* and *trans* isomers in four organisms, including *Ms. trichosporium*, each of which contained $\Delta^x$, $\Delta^9$, $\Delta^{10}$ and $\Delta^{11}$ double bond positional isomers. The double bond positions determined in the previous study were expressed in terms of the total dicarboxylic fragments obtained after permanganate-periodate oxidation of combined 16 and 18 carbon monounsaturated components. Thus, direct comparison of the two analyses is difficult. A higher proportion of *trans* acids was noted in *Ms. trichosporium* by Makula [18] than in our analysis. Variations in the methodology or more probably culture conditions [31,32] may account for this trend.

The fatty acid profile of the type I methylotroph, *Methylomonas* sp., was similar to that reported for *Methylococcus capsulatus* [18,33,34]. A

distinction of type I from type II methylotrophs appears probable based on the higher concentration of 16 carbon monounsaturates in type I bacteria. The separation of methane-utilizing bacteria into two groups based on cellular fatty acids has been reported [35]. Those authors proposed that the groupings showed a good correlation to the membrane type, resting stage and DNA base composition of methane-utilizing bacteria reported by Whittenbury et al. [22].

Methanol-utilizing bacteria have been previously distinguished based on their fatty acid compositions. Bacteria utilizing methanol via the serine pathway (type II) contained between 68-88% of the esterified fatty acid as 18-carbon monoenoics, whilst bacteria using the ribulose monophosphate pathway (type I) contained higher portions of 16-carbon monounsaturates [36]. Although the reasons for the differences in both phospholipid and fatty acid compositions were unknown, the authors suggested that the differences may serve as another criterion for the classification of these bacteria. The distinction of type I and II methylotrophs represents another example of the usefulness of lipids, in particular fatty acids, for the classification of bacteria.

In the past, *trans*-monounsaturated acids have often been reported by organic geochemists in recent marine sediments. It has been proposed that these components originate from microbial and/or abiological degradation of *cis*-mono- and polyunsaturated fatty acids [37-39], or from direct bacterial input [7,8,40]. The presence of significant portions of *trans* monounsaturated acids in a marine-bacterial isolate [41], rumen bacteria [42], *Methylosinus trichosporium*, *Methylococcus capsulatus* [18], and in the methylotrophs analysed in this study is consistent with the view that a direct microbial input of *trans* acids may occur.

The analyses reported here have revealed a number of features. (i) The 3 bacteria studied were readily distinguished, particularly when structural verification of component monounsaturated fatty acids was undertaken. (ii) The distinction of type I and II methylotrophs is possible based on fatty acid compositional data (this study and [18,35]). (iii) LPS β-hydroxy fatty acids were detected in all organisms, further extending the range of Gram-

negative organisms in which these components are found. (iv) Several unidentified cyclic components were found in the neutral lipid fractions of all organisms studied. (v) *trans* Fatty acids were detected in all samples together with several novel *cis* components. Methane-oxidizing bacteria represent a probable source of these specific signature components in sedimentary environments. Methylotrophs are known to participate in the biotransformation of environmental contaminants, such as short chain chlorinated hydrocarbons [43,44]. The data presented here suggest that unique signatures may serve as a screen for these organisms in manipulated laboratory and environmental samples.

## ACKNOWLEDGEMENTS

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# Phospholipid Ester-linked Fatty Acid Biomarkers of Acetate-oxidizing Sulphate-reducers and Other Sulphide-forming Bacteria

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The phospholipid ester-linked fatty acids were examined in four *Desulfobacter* strains (2ac9, AcBa, 3ac10 and 4ac11), a *Desulfobacter*-like 'fat vibrio' (AcKo) and *Desulfotomaculum acetoxidans* (5575), which are all sulphate-reducing bacteria that oxidize acetate. A thermophilic sulphate reducer, *Desulfovibrio thermophilus*, and two sulphur-reducing bacteria, *Desulfuromonas acetoxidans* (11070) and a *Campylobacter*-like spirillum (5175), were also studied. The *Desulfobacter* spp. were characterized by significant quantities of 10-methylhexadecanoic acid. Other 10-methyl fatty acids were also detected in *Desulfobacter* spp. No 10-methyl fatty acids were detected in the other organisms examined, supporting the use of 10-methylhexadecanoic acid as a biomarker for *Desulfobacter*. High levels of cyclopropyl fatty acids, including two isomers of both methylenehexadecanoic (cy17:0) and methyleneheptadecanoic (cy18:0) acids, were also characteristic of *Desulfobacter* spp. The influence of the volatile fatty acids (VFA) propionate, isobutyrate, isovalerate and 2-methylbutyrate on the lipid fatty acid distribution was studied with two *Desulfobacter* strains (2ac9, AcBa) and *Desulfotomaculum acetoxidans*. Although these sulphate reducers cannot oxidize the VFA, their presence in the acetate growth medium caused a shift in the fatty acid distribution in favour of odd-numbered and branched chains by apparent direct incorporation into the fatty acids as chain initiators. The *Desulfobacter* strains were distinguished from other sulphide-forming bacteria by the percentage of unsaturated and the percentage of branched fatty acids.

## INTRODUCTION

Sulphate-reducing bacteria perform the terminal process in anaerobic degradation of organic matter in aquatic environments. These bacteria oxidize low-$M_r$ compounds formed by fermentative bacteria and use sulphate as terminal acceptor, the sulphate being reduced to sulphide. In sulphate-rich habitats such as marine sediments, more than half of the incoming detritus may be mineralized via sulphate reduction (Jorgensen, 1977, 1982; Sansone & Martens, 1982). If sulphate is absent or limiting, as in most freshwater sediments, the terminal degradation step is taken over by methanogenic bacteria and their syntrophic partners (Banat & Nedwell, 1983; Winfrey & Zeikus, 1977; Stieb & Schink, 1985). Molecular hydrogen and acetate are the two key intermediates via which dead biomass (necromass) is channelled into either sulphate reduction or methanogenesis (Loveley *et al.*, 1982; Sorensen *et al.*, 1981; Winfrey & Zeikus, 1977). *Desulfovibrio* spp. and *Desulfobulbus* spp. are potentially hydrogen-scavenging sulphate reducers in nature (Brandis & Thauer, 1981; Kristjansson *et al.*, 1982; Taylor & Parkes, 1985). In the laboratory, *Desulfovibrio* spp. are usually cultivated on lactate (Postgate, 1984), which is probably not a major anaerobic intermediate under natural conditions. Acetate is

oxidized by various sulphate-reducing bacteria (Pfennig *et al.*, 1981), however, only a few species of these, namely *Desulfobacter* spp. and *Desulfotomaculum acetoxidans*, really grow well on acetate (Widdel & Pfennig, 1977, 1981*a, b*) and may, therefore, be significant acetate oxidizers under natural conditions. *Desulfobacter postgatei* exhibits a high affinity for acetate (Schönheit *et al.*, 1982; Ingvorsen *et al.*, 1984). *Desulfobacter* spp. usually do not utilize hydrogen, whereas *Desulfovibrio* spp. and *Desulfobulbus* spp. cannot oxidize acetate. Thus, there are apparently functionally distinctive groups of sulphate-reducing bacteria (Banat *et al.*, 1981).

If elemental sulphur is present, this may be used as terminal acceptor by sulphur-reducing bacteria (Jørgensen, 1982; Smith & Klug, 1981). Mesophilic, eubacterial sulphur reducers that oxidize hydrogen are represented by *Campylobacter*-like spirilla (Wolfe & Pfennig, 1977; Pfennig & Biebl, 1981), and sulphur reducers that oxidize acetate by *Desulfuromonas* spp. (Pfennig & Biebl, 1976, 1981).

Quantitative assay of bacterial biomass and community structure in marine or other sediments requires methods that avoid the problems associated with quantitative recovery of bacteria from surfaces and selective culturing procedures (White, 1983). Recent work has shown that fatty acid analysis may be a powerful tool in the interpretation of microbial community structure (Bobbie & White, 1980) as well as in microbial taxonomy (Minnikin *et al.*, 1978). Few articles detail the lipids of the sulphide-forming eubacteria, most being concerned with the lactate- or hydrogen-utilizing sulphate-reducing bacteria (i.e. *Desulfovibrio* spp. and *Desulfotomaculum* spp.), which commonly exhibit branched monoenoic 17-carbon fatty acids as major components (Makula & Finnerty, 1974, 1975; Ueki & Suto, 1979; Edlund *et al.*, 1985; Boon *et al.*, 1977). Taylor & Parkes (1983) investigated the cellular fatty acids of a *Desulfobacter* sp. (strain 3ac10) utilizing acetate, a *Desulfobulbus* sp. (strain 3pr10) able to utilize propionate, hydrogen or lactate, and a *Desulfovibrio desulfuricans* strain. The *Desulfobacter* strain exhibited high proportions of 10-methylhexadecanoic acid (10Me16:0), which had previously been observed in anoxic marine sediments (Volkman *et al.*, 1980; Parkes & Taylor, 1983; Perry *et al.*, 1979). Because of its absence in *Desulfobulbus* and *Desulfovibrio*, 10Me16:0 was proposed as a biomarker for *Desulfobacter* spp. in these environments. Hitherto, saturated 10-methyl fatty acids have only been observed in actinomycetes and related taxa (Kroppenstedt & Kutzner, 1978).

In this study, we examined other *Desulfobacter* strains for 10Me16:0 to determine if it could be of use as a more general biomarker for that genus. The hydrogen- or lactate-utilizing thermophilic *Desulfovibrio thermophilus* (Rozanova & Khudyakova, 1974) was included in the study in order to compare its phospholipid fatty acids with those of mesophilic *Desulfovibrio* spp. Two representatives of sulphur reducers were also examined: the hydrogen-oxidizing *Campylobacter*-like spirillum 5175 (Wolfe & Pfennig, 1977) and the acetate-oxidizing *Desulfuromonas acetoxidans* (Pfennig & Biebl, 1976). The effects of supplementing the culture medium with volatile fatty acids (not required for growth) on the phospholipid fatty acids of three acetate-utilizing strains were also investigated.

## METHODS

*Bacterial strains and culture methods.* The origins and nutritional capacities of all the strains examined are listed in Table 1.

The 'fat vibrio' strain AcKo is a new isolate from an ammonia-limited freshwater enrichment on acetate. Strain AcKo resembles *Desulfobacter postgatei* in that it uses only acetate as electron donor and its growth is stimulated in brackish media; however, AcKo has motile fat vibrio-shaped cells, whereas *Desulfobacter* cells are oval to rod-shaped and scarcely motile in pure cultures.

The strains were grown in anaerobic 2 litre batch cultures in bicarbonate-buffered media reduced with sulphide and dithionite as described by Pfennig *et al.* (1981). The rubber-sealed culture bottles had a small gas phase of 10–20% (v/v) $CO_2$ in $N_2$. The media were supplemented with vitamins, but contained no yeast extract, chelating agents or redox indicator. For *Desulfovibrio thermophilus*, bicarbonate was omitted and the phosphate concentration was increased from 0.2 to 0.8 g $KH_2PO_4$ l[-1]. The strains required different concentrations of NaCl and $MgCl_2$.$6H_2O$ in their media for optimal growth; the respective concentrations were as follows (g l^{-1}): 1.0 and 0.5 for *Desulfotomaculum acetoxidans*, *Desulfovibrio thermophilus* and spirillum 5175; 7.0 and 1.2 for *Desulfobacter postgatei* and the fat vibrio AcKo; 20.0 and 3.0 for *Desulfuromonas acetoxidans* and the *Desulfobacter* strains AcBa,

Table 1. *Sulphate-reducing and sulphur-reducing bacteria analysed: origin of strains and physiological characteristics*

Culture numbers 1–7 are sulphate-reducing and 8 and 9 are sulphur-reducing bacteria. Growth substrates used in the present study are given in italics.

| Culture no | Strain | DSM no.[a] | Origin | Optimum growth temp. (°C) | Electron donor | Electron acceptors |
|---|---|---|---|---|---|---|
| 1 | *Desulfobacter postgatei* 2ac9 | 2034 | Brackish ditch | 30–32 | *Acetate* | *Sulphate.* sulphite. thiosulphate |
| 2 | *Desulfobacter* sp. AcBa | – | Oil tank | 30–34 | *Acetate* | *Sulphate* |
| 3 | *Desulfobacter* sp. 3ac10 | 2035 | Marine mud | 30–32 | *Acetate,* ethanol | *Sulphate* |
| 4 | *Desulfobacter* sp. 4ac11 | 2057 | Marine mud | 28–31 | *Acetate* | *Sulphate* |
| 5 | 'Fat vibrio' sp. AcKo | – | Sewage ditch | 22–25 | *Acetate* | *Sulphate* |
| 6 | *Desulfotomaculum acetoxidans* 5575 | 771 | Piggery manure | 34–36 | *Acetate,* butyrate. isobutyrate. ethanol | *Sulphate* |
| 7 | *Desulfovibrio thermophilus* | 1276 | Petroleum deposit | 65 | *Pyruvate,* hydrogen, lactate | *Sulphate* |
| 8 | *Desulfuromonas acetoxidans* 11070 | 684 | Marine mud | 30 | *Acetate,* propionate. ethanol | *Malate.* sulphur. fumarate |
| 9 | *Spirillum* 5175 | – | Freshwater mud | 30 | Hydrogen. formate | *Fumarate.* sulphur. malate. nitrate. oxygen (<0·05 atmos.)† |

[a] Deutsche Sammlung von Mikroorganismen. Göttingen. FRG.
† The *Spirillum* may also grow by fumarate or malate fermentation without hydrogen or formate.

Jac10 and Jac11. Media for sulphate reducers contained 25 mM-sulphate. *Desulfovibrio thermophilus* was grown on pyruvate (filter-sterilized, pore size 0.2 µm, final concentration 20 mM). In order to facilitate harvesting of the sulphur reducers, they were grown on alternative purely organic energy sources: spirillum 5175 was cultivated on fumarate (40 mM, gradually added during growth). *Desulfuromonas acetoxidans* on acetate (10 mM) plus malate (40 mM, gradually added). *Desulfobacter postgatei*, *Desulfobacter* strain AcBa and *Desulfotomaculum acetoxidans* were grown in duplicate cultures on acetate, one of each pair containing, in addition, the following VFA (final concentrations in mM): propionate (2), isobutyrate (1), isovalerate (1), 2-methylbutyrate (1). All strains were grown in the dark at their respective optimum temperature (Table 1) and were briefly shaken twice a day. Cells were harvested by centrifugation at the end of exponential growth phase and lyophilized for lipid extraction.

*Lipid extraction and separation.* Lipids were extracted by a modified version (White *et al.*, 1979) of the procedure of Bligh & Dyer (1959). After recovery in chloroform, the lipids were fractionated on a silicic acid column (Gehron & White, 1983). Unisil (1 g, 100–200 mesh) silicic acid (activated at 100 °C, 1 h) was slurried into glass columns with chloroform. Total extractable lipid was then applied to the silicic acid in 2 ml chloroform. Neutral lipids were eluted with 10 ml chloroform, glycolipids with 10 ml acetone and phospholipids with 10 ml methanol. Phospholipid solutions were evaporated to dryness under a stream of nitrogen and stored at −70 °C.

*Methanolysis of phospholipid fatty acids.* Phospholipids were dissolved in 1 ml methanol:toluene (1:1, v/v). 1 ml 0.2 M-KOH was added and the samples were heated at 37 °C for 15 min. Sequential additions of 2 ml hexane, 0.3 ml 1 M-acetic acid and 2 ml water were made. The biphasic mixture was vortexed, and the hexane (upper) layer, containing the fatty acid methyl esters (FAME), was removed. The partition against 2 ml hexane was repeated. Combined hexane fractions were dried under a stream of nitrogen and stored at −70 °C.

*Thin layer chromatography (TLC).* Crude FAME were loaded onto a thin layer silica gel (Whatman KG, 0.25 mm, 20 × 20 cm) plate. Standard methyl nonadecanoate was spotted onto end lanes on each plate. After development of the TLC plates in hexane:diethyl ether (1:1, v/v) the end lanes were sprayed with 0.1% (w/v) Rhodamine to detect the standard. Areas at $R_f$ values corresponding to the standards were scraped off and the FAME eluted from the silica with hexane. The solvent was dried under a stream of nitrogen and the FAME taken up in an appropriate volume of hexane for gas chromatography. Methyl nonadecanoate was added as an internal injection standard.

Saturated and unsaturated FAME were separated on TLC plates as before, but the plates were predeveloped in 20 g AgNO₃, 60 ml water and 120 ml absolute ethanol in the dark. Development and recovery of saturated FAME using an external standard of methyl nonadecanoate was as described above.

*Determination of fatty acid double bond position.* This was done by gas chromatography mass spectrometry (GC–MS) analysis of their Diels–Alder adducts (Kidwell & Bieman, 1982), as modified by Nichols *et al.* (1985), or of dimethyl disulphide derivatives (Dunkelblum *et al.*, 1985).

*Determination of fatty acid cyclopropyl position.* This procedure was modified from McCloskey & Law (1967), and Kaneshiro & Marr (1961). Cyclopropyl FAME (approximately 5 mg) were hydrogenated in the presence of PtO₂ (2 mg) and glacial acetic acid (0.2 ml), under 275 kPa H₂, while stirring with a glass coated magnetic stirring bar for 20 h. The solvent was removed under nitrogen, and the FAME were analysed by GC–MS.

*GC and GC–MS.* Gas chromatography was done on a Varian model 3700 using a flame ionization detector and a non-polar cross-linked methyl silicone fused silica capillary column (50 m × 0.2 mm i.d., Hewlett Packard). Injector and detector temperatures were 250 °C and 290 °C respectively. The oven temperature was programmed from 80 °C to 140 °C at 20 °C min⁻¹, then at 4 °C min⁻¹ to 270 °C, and then isothermal for 10 min. Hydrogen was used as carrier gas at 30 cm sec⁻¹ (linear velocity). Peak areas were integrated by a Hewlett Packard 3350 series programmable laboratory data system operated in an internal standard programme mode.

Initial GC–MS analysis was done on a Hewlett Packard 5995A system fitted with a direct capillary inlet (Nichols *et al.*, 1985). Several samples were also analysed with the following GC–MS parameters: splitless injection at 100 °C; the oven programmed from 100 °C to 300 °C at 4 °C min⁻¹; helium as the carrier gas; electron multiplier 1300–1400 V; transfer line 300 °C; source and analyser 250 °C, autotune file DFTPP normalized; optics tuned at *m/z* 502; MS peak threshold = 100 triggered on total ion abundance; electron impact energy = 70 eV. Mass spectral data were processed with a Hewlett Packard RTE 6 VM data system.

*Criteria for the identification of FAME.* Identification of individual fatty acids relied upon GC retention time; argentation TLC separation of unsaturated and saturated FAME; derivatization of monoenoic acids for the determination of double bond positions; and the hydrogenation of cyclopropyl groups for mass spectral analysis of the resultant branched FAME. Original FAME were also examined by GC–MS. Interpretation of mass spectral data and component identification was achieved by comparison with published data (Dinh-Nguyen *et al.*, 1961, Ryhage & Stenhagen, 1958, Rohwedder *et al.*, 1965, Campbell & Naworal, 1969).

## RESULTS

The phospholipid ester-linked fatty acid distributions of the *Desulfobacter* spp. and of all the other sulphide-forming bacteria analysed are listed in Tables 2 and 3, respectively. For ease of

**Table 2.** *Percentage phospholipid ester-linked fatty acids of Desulfobacter spp. grown on acetate*

Cultures are numbered according to Table 1. Fatty acids are characterized by chain length and position of substituents from the methyl ($\omega$) end. Thus $16:1\omega7c$ is $\omega7$-cis-hexadecenoic acid. Iso and anteiso FAME are methyl-branched one and two carbons from the methyl end, respectively. Unsaturation may occur in either cis (c) or trans (t) configurations. Other notation: tr, trace quantities ($<0.1\%$); br, multiple branching; SAT, unknown saturated fatty acid; *a*, FAME detected by double bond analysis only, and not quantified; •, positional data for double bond, methyl branch or cyclopropyl ring (cy) based on GC retention time only (insufficient sample was available for analysis). Cultures + VFA were grown with additional volatile fatty acid (final concentration mM): propionate (2), isobutyrate (1), isovalerate (1), 2-methylbutyrate (1)

| FAME | 1 | 1 + VFA | 2 | 2 + VFA | 3 | 4 |
|---|---|---|---|---|---|---|
| 13:0 | | | tr | | tr | |
| iso14:0 | tr | 0.2 | 0.1 | 1.1 | 0.2 | tr |
| anteiso14:0 | | | | 4.5 | | |
| 14:1ω7 | 0.3 | 0.2 | 0.4 | | 0.3 | tr |
| 14:1ω5 | | | tr | | | tr |
| 14:0 | 9.5 | 2.4 | 31.4 | 9.4 | 8.8 | 11.3 |
| br15:1• | | 0.8 | tr | | 0.8 | tr |
| iso15:0 | 1.1 | 8.9 | 1.6 | 2.6 | 8.7 | 2.1 |
| anteiso15:0 | 0.1 | 4.0 | 0.1 | 1.9 | 0.2 | 0.1 |
| 15:1ω8 | | | | *a* | | *a* |
| 15:1ω6 | | 1.5 | 0.1 | 4.6 | tr | |
| 15:1• | | | | 0.2 | | tr |
| 15:0 | 1.3 | 12.1 | 0.7 | 23.7 | 0.8 | 0.4 |
| 10Me15:0 | | 1.4 | | | | |
| br16:1ω6c• | | | | 1.9 | 0.3 | |
| iso16:0 | 0.1 | 1.2 | 0.4 | 0.9 | 0.6 | 0.2 |
| anteiso16:0 | | | | 5.3 | | |
| 16:1ω9 | 1.4 | 0.6 | 0.9 | | 1.4 | 0.3 |
| 16:1ω7c | 4.9 | 2.1 | 5.9 | 1.8 | 7.1 | 1.1 |
| 16:1ω7t | 0.3 | | 0.2 | | 0.2 | 0.1 |
| 16:1ω5 | 2.0 | 1.3 | 1.8 | 6.3 | 1.8 | 1.4 |
| cy16:0• | | 1.8 | | | | |
| 16:0 | 30.7 | 11.9 | 18.6 | 7.4 | 25.8 | 32.7 |
| iso17:1ω7 | 0.1 | 1.5 | 0.1 | 0.1 | 1.4 | 0.2 |
| 10Me16:0 | 16.5 | 9.1 | 9.4 | 6.3 | 24.7 | 11.2 |
| iso17:0 | 0.2 | 1.3 | tr | tr | 1.4 | 0.2 |
| anteiso17:0 | 0.2 | 1.3 | | | 0.1 | |
| 17:1ω8 | | 2.0 | | 0.7 | | *a* |
| 17:1ω7 | *a* | | | | | *a* |
| 17:1ω6 | *a* | 8.9 | | 3.6 | | *a* |
| cy17:0a(ω7/8) | 24.4 | 10.0 | 27.3 | 11.8 | 7.0 | 30.1 |
| cy17:0b• | 2.6 | 0.8 | | 0.2 | | 2.6 |
| brSAT• | | | 0.5 | | | |
| 17:0 | 0.2 | 2.9 | tr | 1.0 | 0.2 | 0.2 |
| br18:1• | 0.2 | 1.4 | | 0.3 | tr | tr |
| 10Me17:0 | 0.1 | 2.4 | | 2.1 | 0.4 | 0.1 |
| 18:1ω9 | 0.2 | 0.2 | tr | tr | 0.4 | 0.1 |
| 18:1ω7c | 0.6 | 0.5 | 0.1 | | 3.8 | 0.4 |
| 18:1ω7t | 0.1 | | | | tr | |
| cy18:0a• | | 0.8 | | 0.9 | | 0.1 |
| cy18:0b• | 0.4 | 4.5 | | 0.8 | | 0.2 |
| 18:0 | 0.3 | 0.3 | 0.1 | 0.4 | 0.3 | 0.2 |
| br19:1• | 0.8 | 0.4 | tr | | 1.6 | 1.0 |
| 10Me18:0 | | | | | 0.5 | |
| anteiso19:0 | 0.1 | 0.1 | tr | | tr | tr |
| cy19:0• | 1.1 | 0.6 | 0.2 | | 0.4 | 1.4 |
| Trace components | 0.2 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 |
| Branched FAME | 19.5 | 14.5 | 12.2 | 27.0 | 41.5 | 15.1 |
| Unsaturated FAME | 10.9 | 21.4 | 9.5 | 19.5 | 19.1 | 4.6 |
| μmol (g dry wt)$^{-1}$ | 102.5 | 77.3 | 58.5 | 40.8 | 81.5 | 64.4 |

**Table 3.** *Percentage phospholipid ester-linked fatty acids of a Desulfobacter-like 'Fat vibrio', non-Desulfobacter sulphate reducers and sulphur reducers*

For definition of abbreviations see Table 2

| | | | Culture no | | | |
|---|---|---|---|---|---|---|
| FAME | 5 | 6 | 6 + VFA | 7 | 8 | 9 |
| iso14:0 | tr | | 0.3 | | | |
| 14:1ω7 | 0.2 | | | | 0.1 | 0.3 |
| 14:1• | 0.1 | | | | 0.1 | |
| 14:0 | 5.5 | 1.3 | 0.6 | | 2.1 | 3.1 |
| br15:1• | 0.3 | | | | tr | |
| iso15:0 | 3.5 | 0.5 | 0.9 | 0.3 | 4.4 | tr |
| anteiso15:0 | tr | | | 0.2 | 0.4 | tr |
| 15:1ω8 | - | | a | | | |
| 15:1ω6 | tr | | 0.4 | | 0.8 | 0.1 |
| 15:1ω5 | | | a | | | a |
| 15:0 | 0.1 | 0.3 | 4.5 | | | 1.0 |
| br16:1ω6• | tr | - | 3.2 | | | |
| iso16:0 | 0.2 | | 2.0 | 23.4 | 0.1 | |
| 16:1ω9 | 0.8 | 5.9 | 3.5 | | | |
| 16:1ω7c | 23.9 | 24.4 | 13.4 | | 37.3 | 50.1 |
| 16:1ω7t | 0.4 | 0.6 | tr | | 0.5 | 1.1 |
| 16:1ω5 | 1.6 | 4.8 | 2.6 | | 2.6 | 1.3 |
| 16:0 | 41.5 | 34.0 | 22.2 | 4.1 | 47.3 | 31.8 |
| iso17:1ω7 | 1.0 | - | - | | 0.2 | - |
| iso17:0 | 0.6 | - | - | 9.6 | 0.7 | - |
| anteiso17:0 | tr | | | 39.6 | | |
| 17:1ω8 | - | | 5.2 | - | 0.4 | tr |
| 17:1ω6 | - | - | 13.3 | | 0.6 | 0.5 |
| cy17:0a(ω7/8) | 18.2 | | - | | | - |
| cy17:0b• | 1.1 | | | | | |
| 17:0 | tr | - | 7.0 | 0.8 | 0.8 | 0.2 |
| iso18:0 | | | | 12.9 | | |
| 18:1ω9 | 0.1 | 1.0 | 0.9 | | tr | |
| 18:1ω7c | 0.5 | 24.1 | 17.0 | | 0.8 | 9.7 |
| 18:1ω7t | - | 0.4 | | | | 0.2 |
| 18:1ω5 | | 1.4 | 0.9 | | tr | 0.1 |
| 18:0 | tr | 1.2 | 1.2 | 7.7 | 0.4 | 0.2 |
| iso19:0 | | | | 0.3 | | |
| anteiso19:0 | tr | | 0.8 | 0.9 | 0.2 | tr |
| cy19:0• | 0.3 | | | | | |
| Trace components | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 |
| Branched FAME | 5.6 | 0.5 | 7.2 | 87.2 | 6.0 | tr |
| Unsaturated FAME | 28.9 | 62.6 | 60.4 | 0.0 | 43.4 | 63.4 |
| μmol (g dry wt)⁻¹ | 81.5 | 19.8 | 9.8 | 13.8 | 61.5 | 49.2 |

interpretation of major differences, the total percentages of branched and unsaturated fatty acids occurring in each strain are presented at the bottom of each table. Due to the constraints of time and materials only one batch culture of each strain and set of conditions was analysed. Previously this laboratory has shown that phospholipid fatty acid profiles of duplicate batch cultured bacteria usually have a standard deviation of less than 1% for each fatty acid (e.g. Edlund *et al.*, 1985).

The sulphide-forming bacteria in this study are clearly distinguished by the presence or absence of 10-methyl fatty acids. Among the strains examined, 10-methyl fatty acids occur exclusively and consistently in the *Desulfobacter* spp. In *Desulfobacter* spp. grown on acetate, 10Me16:0 ranged from 24·7% in *Desulfobacter* sp. 3ac10 to 9·4% in *Desulfobacter* sp. AcBa.

VFA were added to cultures of *Desulfobacter postgatei* 2ac9, *Desulfobacter* sp. AcBa and *Desulfotomaculum acetoxidans* 5575. In these cultures, all even-numbered fatty acids were
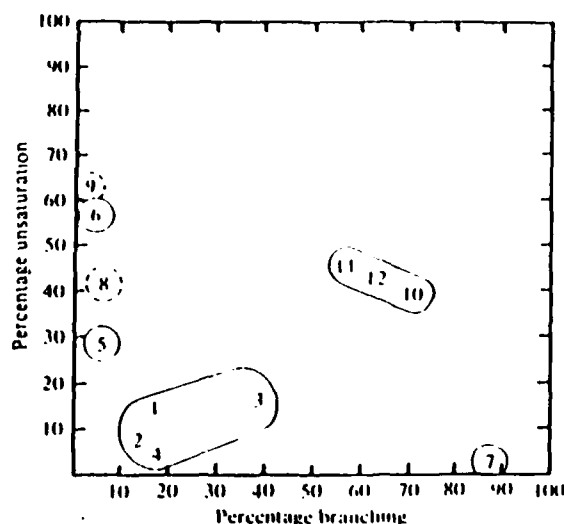
Fig. 1. Graph illustrating the distribution of the sulphidogenic bacteria based on percentage unsaturation and percentage branching of their fatty acids. Numbers refer to the culture numbers in Table 1 except for: 10, *Desulfovibrio desulfuricans*; 11, *Desulfotobrio vulgaris*, 12, *Desulfovibrio africanus*. Strains 10–12 are shown for reference; the data for them is from Edlund *et al.* (1985), by kind permission of the authors. Broken lines: sulphur-reducing bacteria

present in decreased proportions. Those of strain 2ac9 decreased from 67 to 39% with respect to the total, those of strain AcBa from 69 to 47% and those of strain 5575 from 99 to 67%. Simultaneously the branched chain fatty acids increased (2ac9, from 19 to 34%; AcBa, from 12 to 27%; 5575, from 0·5 to 7%).

All the *Desulfobacter* spp. grown on acetate were characterized by even-numbered fatty acid distributions ranging from 77% (3ac10) to 59% (4ac11). 10-Methyl fatty acids other than 10Me16:0 were detected, including 10Me15:0 (2ac9 + VFA), 10Me17:0 (2ac9 ± VFA; AcBa + VFA; 3ac10 − VFA; 4ac11 − VFA) and 10Me18:0 (3ac10 − VFA). Components in insufficient quantity for structural confirmation by GC-MS (2ac9 − VFA; AcBa + VFA; 3ac10) had identical retention times to 10Me15:0; these components are not listed in Table 2.

Cyclopropyl fatty acids are also characteristic of *Desulfobacter* spp. The major component was ω7/ω8 methylenehexadecanoic acid (cy17:0a), which ranged from 30% in 4ac11 to 7% in 3ac10 when the bacteria were grown on acetate without the VFA. The position of the cyclopropyl group in a second methylenehexadecanoic acid (cy17:0b) was not identified; this fatty acid ranged from 0% in AcBa to 3% in 4ac11 and 2ac9 under the same conditions. *Desulfobacter* spp. 2ac9 and AcBa showed a decrease in proportion of cyclopropyl fatty acids when VFA were included in the media (2ac9, from 28 to 18%; AcBa, from 27 to 13%). Other cyclopropyl FAME were also detected including cy16:0 (2ac9 + VFA) and two cyclopropyl 18:0 FAME designated cy18:0a and cy18:0b.

The 'fat vibrio' strain AcKo grown on acetate had 75% even-numbered and 94% straight-chain fatty acids, with high levels of cy17:0 (cy17:0a, 18%; cy17:0b, 1%) similar to the *Desulfobacter* spp.; however, no 10-methyl FAME could be detected.

*Desulfotomaculum acetoxidans* 5575 grown on acetate gave a distribution of 99% even-numbered and 99% straight-chain fatty acids. Incorporation of VFA into the medium decreased the proportions of even-numbered and straight-chain fatty acids to 68 and 93%, respectively. High levels of 16:1ω7c (24%) and 18:1ω7c (24%) were characteristic of this organism. Monoenoic fatty acids represented 62% (− VFA) and 60% (+ VFA) of the total. Neither 10-methyl nor cyclopropyl FAME were detected.

*Desulfovibrio thermophilus* grown on pyruvate had no detectable unsaturated fatty acids. Odd-numbered and branched-chain fatty acids predominated (51 and 87% respectively) in this

species, in contrast to the other organisms. The major fatty acids were iso16:0 and anteiso17:0.

*Desulfuromonas acetoxidans* 11070, a sulphur-reducing bacterium grown on acetate plus fumarate, exhibited a distribution of 91% even-numbered and 94% straight-chain fatty acids. This organism showed the highest proportions of the fatty acids 16:0 (47%) and 16:1ω7c (37%), which are common among all the strains examined except the *Desulfovibrio thermophilus* strain, which lacked 16:1ω7c; otherwise, no distinctive components were detected.

The *Campylobacter*-like spirillum strain 5175, which also utilized elemental sulphur as an electron acceptor, was grown on fumarate. It had a distribution of predominantly even-numbered (98%) and straight-chain (99%) fatty acids. The major fatty acids were 16:1ω7c (50%) and 16:0 (32%).

Fig. 1 shows a graphic representation of all the organisms examined based on the percentage unsaturation and percentage branching in their fatty acids. Organisms showing homology of morphological and physiological parameters (e.g. the *Desulfobacter* spp.) are grouped. The sulphur-reducing bacteria (*Desulfuromonas* and the spirillum) are encircled with broken lines to distinguish them from the sulphate-reducing bacteria. No other homology of characters in the sulphur reducers is implied.

### DISCUSSION

All the strains examined could be differentiated by their respective distributions of phospholipid ester-linked fatty acids.

The *Desulfobacter* spp. exhibited homology of fatty acid biomarkers. High levels of cyclopropyl and 10Me16:0 fatty acids were observed with largely an even-numbered, straight-chain fatty acid distribution after growth on acetate. This is in agreement with Taylor & Parkes (1983), who examined the fatty acids of *Desulfobacter* sp. 3ac10. Our profile for 3ac10 also included low levels of 10Me17:0 and 10Me18:0 (Table 2). These unusual fatty acids, as well as 10Me15:0, were also found in other *Desulfobacter* spp. No other genera contained 10-methyl fatty acids. Kroppenstedt & Kutzner (1978) showed that 10-methyl fatty acids occur in actinomycetes and increase as a function of age; however, unlike *Desulfobacter* spp., 10Me17:0 and 10Me18:0 tend to dominate in these filamentous bacteria.

Some bacterial fatty acid profiles vary in composition according to external stimuli (temperature, pH, nitrogen source, salinity, etc.; Lechevalier, 1976). In order to use specific fatty acid biomarkers to interpret environmental community structure, micro-organisms should be examined for fatty acid patterns and their variation under different conditions. Taylor & Parkes (1983) showed that fatty acid profiles in some sulphate-reducing bacteria can be influenced by carbon source; however, in all cases major fatty acid biomarkers were identifiable. With three separate cultures of *Desulfobulbus* sp. 3pr10 it was shown that growth on propionate produced an odd carbon number straight-chain profile, $CO_2/H_2$ produced an even carbon number straight-chain profile and lactate an approximately equal distribution of odd and even carbon number and of straight and branched chains. These changes could be explained in terms of the carbon sources being used as fatty acid chain initiators. This study is concerned with the induced variation in fatty acid profiles due to the presence of propionate and branched-chain volatile fatty acids. The latter are formed in sediments and sludges by fermentative breakdown of branched-chain amino acids. With the exception of isobutyrate being oxidized by *Desulfotomaculum acetoxidans* (Widdel & Pfennig, 1981b), none of the added VFA could be utilized as electron donor by the sulphate reducers. The present study has shown that interpretation of environmental data using lipids should consider the potential effect of a variety of VFA produced by fermentative bacteria, which may change the fatty acid distributions of various micro-organisms present. Ingram *et al.* (1977) showed that exogenous propionate can be incorporated as a chain initiator into *Escherichia coli* fatty acids during growth on glucose broth, giving rise to odd-numbered chains. It seems likely that the VFA incorporated into strains 2ac9, AcBa and 5575 were used as chain initiators, because all three cultures displayed increased proportions of odd carbon number and branched fatty acids (see Tables 2 and 3). *Desulfobacter* sp. AcBa grown in media containing VFA synthesized anteiso14:0 and anteiso16:0; the

presence of these fatty acids could not be interpreted in terms of any of the VFA being used as chain initiator (Kaneda, 1977)

Carbon sources influenced 10-methyl fatty acids and their straight-chain equivalents in similar ways; their proportion was lowered when odd-numbered and branched VFA were incorporated into the growth medium. As Kroppenstedt & Kutzner (1978) suggest, this implies that 10Me16:0 is formed by methylation at the 10th carbon after initial synthesis of a mono-unsaturated 16-carbon acid. If so, this almost certainly takes place *in situ* to the esterified phospholipid in the membrane (Akamatsu & Law, 1970)

The 'fat vibrio' strain AcKo exhibits both nutritional and fatty acid profile similarities to *Desulfobacter* spp., however, strain AcKo has no 10-methyl fatty acids

*Desulfotomaculum acetoxidans* 5575 grown on acetate exhibited a very different fatty acid distribution compared with that of the *Desulfobacter* spp. or the 'fat vibrio' AcKo. Neither cyclopropyl nor 10-methyl fatty acids were detected. The predominant fatty acids were 16:1ω7c, 18:1ω7c and 16:0. The presence of substantial amounts of unsaturated fatty acid in *Desulfotomaculum acetoxidans* (62",), *Desulfuromonas acetoxidans* (44",) and the *Campylobacter*-like spirillum (63",), as compared to the other sulphide-forming bacteria, probably does not indicate a physiological or genealogical relationship. Nutritionally, these species are very different; moreover, the sporogenous *Desulfotomaculum acetoxidans* belongs to the *Clostridium* branch of the Gram-positive bacteria, whereas *Desulfuromonas* and the spirillum are Gram-negative (Fowler *et al.*, 1985). Bacteria actually assigned to the genus *Campylobacter* exhibit high levels of unsaturation (approximately 50",), with no branched fatty acids (Blaser *et al.*, 1980), and have fatty acid profiles that are similar to that of the *Campylobacter*-like spirillum 5175 (although strain 5175 exhibited some branched fatty acids).

*Desulfovibrio thermophilus*, unlike other *Desulfovibrio* spp. (Edlund *et al.*, 1985), exhibited no detectable unsaturated fatty acids; however, 87", were iso or anteiso branched. Silvius & McElhaney (1979a, b) have shown that iso and anteiso branching provide the same effect as unsaturation with respect to membrane fluidity. This could explain why some bacteria have a low degree of fatty acid branching with high unsaturation and others have completely the reverse. Thus the *Campylobacter*-like spirillum and *Desulfovibrio thermophilus* may achieve the same effect by different mechanisms.

The ratios of unsaturation to branching of the fatty acids of different sulphide-forming bacteria can be used to distinguish certain physiological and taxonomic groups (Fig. 1). This does not apply to *Desulfotomaculum acetoxidans* and the sulphur-reducing spirillum, but the presence or absence of certain fatty acids (Table 3) can be used to separate these two organisms.

In this study we have shown that among the sulphide-forming bacteria examined only members of the genus *Desulfobacter* (all acetate utilizers) contain the fatty acid 10Me16:0. Other genera containing 10Me16:0 in their fatty acids include members of the actinomycetales (Kroppenstedt & Kutzner, 1978). However, those members which do exhibit 10-methyl fatty acids have major quantities of 10Me18:0, but not of 10Me16:0. Preliminary attempts to assess the contribution of these actinomycetes to the microbial biomass of marine sediments appear to indicate that they are terrestrial organisms that have been washed into the sea, where they do not contribute to biomass turnover (Goodfellow & Haynes, 1984). Thus the presence of 10Me16:0 and cy17:0, without high levels of 10Me18:0, seems to have the potential to act as a biomarker for *Desulfobacter* spp. in marine sulphate-reducing environments.

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